

**CLINICAL AND MOLECULAR CHARACTERIZATION
OF MONOGENIC DIABETES MELLITUS
IN INFANTS AND YOUNG CHILDREN**



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To all children with diabetes;
they know better than anybody else what this all is about.

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List of Abbreviations

DBD: DNA-binding domain

GADA: glutamic acid decarboxylase antibodies

HbA1c: glycated hemoglobin

HLA: human leukocyte antigen

IA2A: insulinoma associated antigen-2 antibodies

IAA: insulin autoantibodies

ICA: islet cell autoantibodies

IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked

IQ: intelligence quotient

IQR: interquartile range

JDF: juvenile diabetes foundation

MODY: maturity-onset diabetes of the young

MRI: magnetic resonance imaging

PNDM: permanent neonatal diabetes

TNDM: transient neonatal diabetes

AGPAT2: 1-acylglycerol-3-phosphate O-acyltransferase 2

BSCL: Berardinelli-Seip congenital lipodystrophy (seipin)

LMNA: lamin A/C

PPARG: peroxisome proliferator-activated receptor- γ

ZMPSTE24: zinc metalloproteinase Ste24

AKT2: V-AKT thymoma viral oncogene homolog 2

CIDEC: cell death-inducing DFFA-like effector C

ALMS1: Ålstrom syndrome 1

BBS: Bardet-Biedl syndrome

K_{ATP} channel: ATP-sensitive potassium channel

DEND: developmental delay, epilepsy, neonatal diabetes

ZAC/PLAGL1: pleomorphic adenoma gene-like 1

IPF1/PDX1: insulin promoter factor 1

PTF1A: pancreas transcription factor 1, alpha subunit

HNF1B: hepatocyte nuclear factor-1-beta

GLIS3: GLIS family zinc finger protein 3

KCNJ11: potassium channel, inwardly rectifying, subfamily J, member 11

ABCC8: ATP-binding cassette, subfamily C, member 8

GCK: glucokinase

SLC2A2: solute carrier family 2 (facilitated glucose transporter), member 2

GLUT2: glucose transporter 2

SLC19A2: solute carrier family 19 (thiamine transporter), member 2

INS: insulin

EIF2AK3: eukaryotic translation initiation factor 2-alpha kinase 3

FOXP3: forkhead box P3

HNF4A: hepatocyte nuclear factor-4-alpha

HNF1A: hepatocyte nuclear factor-1-alpha

NEUROD1: neurogenic differentiation 1

OGTT: oral glucose tolerance test

HDL: high density lipoprotein

LDL: low density lipoprotein

KLF11: Kruppel-like factor 11

PAX4: paired box gene 4

ISL1: islet 1

RCAD: renal cysts and diabetes

DIDMOAD: diabetes insipidus, diabetes mellitus, optic atrophy, deafness

WFS1: Wolfram syndrome 1

CISD2: CDGSH iron sulfur domain protein 2

MIDD: maternally-inherited diabetes and deafness

MELAS: mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes

TRMA: thiamine-responsive megaloblastic anemia

THTR1: thiamine transporter protein 1

CEL: carboxyl-ester lipase

CFTR: cystic fibrosis transmembrane conductance regulator

PRSS1: protease, serine, 1

SPINK1: serine protease inhibitor, Kazal-type, 1

INSR: insulin receptor

rhIGF-I: recombinant human insulin-like growth factor I

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CHAPTER 1

Introduction, Rationale and Objectives

1.1 BACKGROUND

Diabetes mellitus is one of the most frequent chronic diseases in children and adolescents (Sperling, 1996). It is not a single disease but a group of metabolic disorders characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Ize-Ludlow et al, 2005). Until recently, childhood diabetes has not been considered a diagnostic specialty, as patients were almost undoubtedly considered to have type 1 diabetes. However, this assumption is no longer correct. Although insulin-deficient diabetes is by far the commonest subtype of diabetes in childhood and autoimmune type 1 diabetes accounts for the vast majority of cases (>95%), an increasing body of evidence demonstrates that several other types of diabetes can manifest during the first two decades of life (Porter et al, 2004). This fact has important implications for clinical practice, as non-type 1 diabetes differs from type 1 diabetes not only in its cause, but also in its prognosis and associated conditions, and the cause of diabetes may even guide the most appropriate treatment. This has proved to be true especially for certain subtypes of monogenic diabetes, which represents 1-2% of all diabetes cases.

1.2 GENETICS OF DIABETES MELLITUS

Genes play an important role in the development of diabetes, whichever type is considered. From a genetic point of view, diabetes can be classified as being polygenic or monogenic. Overall, polygenic diabetes accounts for the vast majority of cases both in children and in adults. The study of the genetics of polygenic diabetes is fraught with difficulty because there are multiple predisposing polymorphisms, each having a small effect, while environmental factors also play a large role (Gill-Carey et al, 2007). In contrast, monogenic diabetes is rare and results from the presence of one or more

mutations in a single gene. Almost all cases of monogenic diabetes in children and adolescents result from mutations in genes causing β -cell loss or β -cell dysfunction and hence affect insulin synthesis, packaging, glucose sensing or insulin secretion, although diabetes can rarely occur from mutations resulting in very severe insulin resistance (Hattersley et al, 2009).

1.3 POLYGENIC DIABETES

In most western countries, type 1 diabetes accounts for more than 95% of childhood and adolescence diabetes (Thunander et al, 2008). Type 2 diabetes is becoming more common and, although rare in unselected pediatric diabetic patients as a whole, it accounts for a significant proportion of youth-onset diabetes in certain at-risk populations (Shaw, 2007).

1.3.1 Type 1 diabetes

Type 1 diabetes results from selective destruction of the pancreatic insulin-producing β -cells, which occurs at a variable rate and becomes clinically symptomatic when approximately 90% of β -cells has been destroyed (Gillespie, 2006). The β -cells are destroyed by an autoimmune response mediated by T cells that react specifically to one or more β -cell proteins. Serological markers of the autoimmune process, including islet cell (ICA), glutamic acid decarboxylase (GADA), islet antigen-2 (IA2A), or insulin autoantibodies (IAA), are present in 85–90% of affected children when fasting hyperglycemia is detected (Sabbah et al, 1996). However, whether they participate in the pathogenic mechanism of the disease remains to be fully understood. When the clinical presentation is typical of type 1 diabetes, often associated with diabetic ketoacidosis

(Roche et al, 2005), but antibodies are absent, then the diabetes is classified as type 1b or idiopathic (Tiberti et al, 2000). The environmental triggers that initiate pancreatic β -cell destruction remain largely unknown, but the process usually begins months to years before the manifestation of clinical symptoms.

Familial aggregation of type 1 diabetes has been recognized for a long time, and ~10–13% of newly diagnosed children have an affected first-degree relative, this proportion being higher for siblings of affected patients than for offspring or parents. The risk of diabetes mellitus to an identical twin of a patient with type 1 diabetes is about 36% and for a sibling the risk is approximately 4% by the age of 20 years and 9.6% by the age of 60 years, compared with 0.5% for the general population. The younger the proband is at diagnosis, the higher the risk for siblings. Type 1 diabetes is two to three times more common in the offspring of diabetic men (3.6–8.5%) compared with diabetic women (1.3–3.6%) (Marcovecchio et al, 2007).

Despite this familial clustering, there is no recognizable pattern of inheritance of the disease. Instead, association studies exploiting unbiased genome-wide analyses have identified over 40 regions in the human genome that are linked to type 1 diabetes, but most make only a minor contribution overall to the genetic susceptibility to this disease (Barrett et al, 2009; Concannon et al, 2009). The most significant susceptibility locus (*IDDM1* locus) is the HLA class II region in the major histocompatibility complex on chromosome 6p21.3, as allelic variation within this locus contributes about 50% of the inherited risk for type 1 diabetes. The disease susceptibility conferred by HLA represents the combined effect of several genes within the region, *DRB1* and *DQB1* genes being the major determinants of HLA-encoded susceptibility to type 1 diabetes. Whereas several HLA genotypes confer increased risk, other genotypes confer protection. In Caucasians, type 1 diabetes is strongly associated with HLA DR3-DQ2 and DR4-DQ8 haplotypes, and recent studies from different European countries have confirmed that the HLA DR3-DQ2/DR4-DQ8 genotype is associated with the highest diabetes risk. This genotype is found in 20–30% of type 1 diabetic patients and in almost 50% of patients diagnosed in

early childhood. In contrast, genotypes containing the HLA DQ6 haplotype confer dominant protection from type 1 diabetes. Several other disease susceptibility loci have been clearly demonstrated based on their direct effect on risk. However, a large proportion of type 1 diabetes clustering remains unexplained (Bartsocas et al, 2006).

More than half of individuals with type 1 diabetes are diagnosed before the age of 15 years. A well documented rise in the incidence of approximately 3% per year has been noted in many countries (DIAMOND Project Group, 2006), and in some reports, there has been a disproportionately greater increase in those less than 5 years. As a rule of thumb, the earlier the onset of the disease, the stronger the HLA-defined genetic susceptibility and the more frequent an autoantibody response is detected (Komulainen et al, 1999). Nevertheless, among those type 1 diabetes patients diagnosed during childhood, less than 5% present clinically within the first 2 years of life. Moreover, because months or even years can elapse between the beginning of the autoimmune response and the clinical appearance of type 1 diabetes, the onset of diabetes very early in life might not be consistent with the time required for an autoimmune response to result in overt diabetes, even if the autoimmune disease begins in the fetal period (Lindberg et al, 1999; Achenbach et al, 2005). Thus, clinical presentation of type 1 diabetes in infancy is rare, especially within the first 6 months of life. Children diagnosed after the sixth month of life have an HLA-DQA1 and DQB1 genotype distribution similar to older classic type 1 diabetic patients, whereas “early onset” diabetic children are similar to control subjects (Figure 1.1) (Iafusco et al, 2002; Edghill et al, 2006). These data are even more relevant if we consider that type 1 diabetic patients diagnosed in childhood are more likely than adults to carry HLA alleles associated with disease susceptibility and, in addition, in very young children diabetes appears to be associated with a higher-risk genotype than in older children. Individuals diagnosed before 6 months of age are therefore unlikely to have polygenic autoimmune type 1 diabetes. However, it is difficult to be certain of an absolute cut-off at 6 months. The fact that the prevalence of high-risk HLA is slightly lower in those diagnosed between 6 and 12 months of age compared with those between

12 and 24 months of age indicates that there may be a few patients with non-autoimmune diabetes in the 6- to 12-month age range (Figure 1.1) (Edghill et al, 2006).

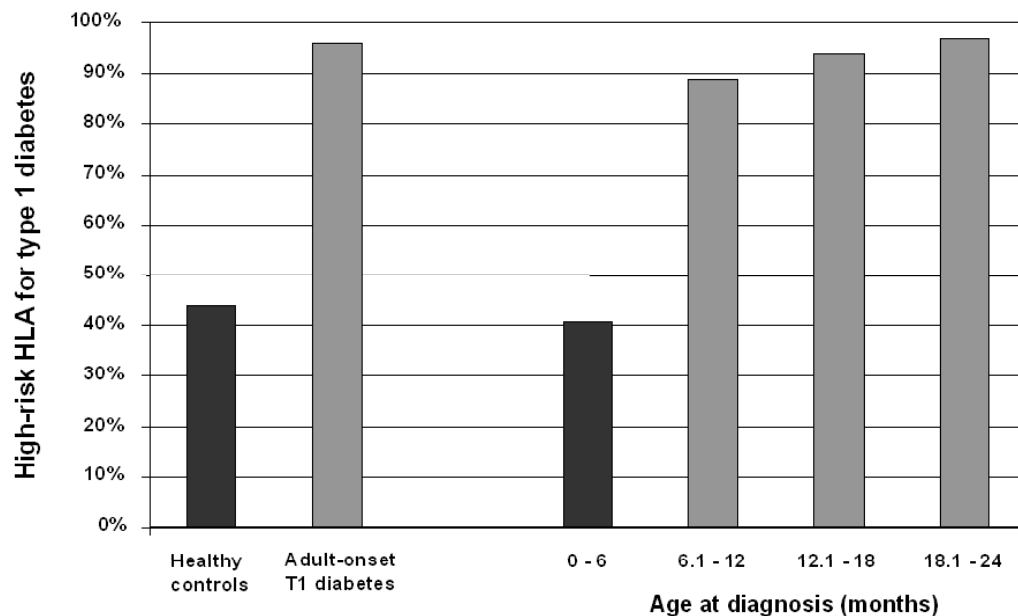


Figure 1.1. Prevalence of high-risk HLA class II genotypes (DR4-DQ8/DR3-DQ2, DR4-DQ8/X, and DR3-DQ2/X, where X is not DR2-DQ6) in subjects diagnosed with diabetes under 24 months of age, compared to patients with adult-onset type 1 diabetes and normal controls (modified with permission from Edghill et al, 2006).

1.3.2 Type 2 diabetes

Type 2 diabetes is often considered a polygenic disorder with multiple genes located on different chromosomes being associated with this condition. This is further complicated by numerous environmental factors that also contribute to the clinical manifestation of the disorder in genetically predisposed persons (Porter et al, 2007). Once considered almost restricted to adults, the incidence of type 2 diabetes is increasing among pediatric patients (Shaw, 2007). The rapid increase in the population prevalence of type 2 diabetes in youth can only be explained by changes in lifestyle, as it parallels the increasing incidence of childhood obesity worldwide. However, even in

sedentary western culture, only a small minority of obese children develops type 2 diabetes. Genetic factors are important in determining the children who become obese and also the obese children who develop type 2 diabetes (Gill-Carey et al, 2007). Support for the role of genetic factors comes from epidemiological evidence that type 2 diabetes in youth is most common in individuals from racial groups with a high prevalence of diabetes and in subjects with a strong family history. *TCF7L2* has recently been discovered as the gene with the greatest impact on the risk of type 2 diabetes at least in the European populations (Grant et al, 2006; Groves et al, 2006; Scott et al, 2006; Florez et al, 2006; Cauchi et al, 2006; van Vliet-Ostaptchouk et al, 2007). However, systematic population screening for type 2 diabetes cannot be justified in most countries because of the low prevalence. Nevertheless, targeted screening is recommended if youths are overweight and have any two risk factor listed in Table 1.1, starting at 10 years or at onset of puberty, whichever is earlier, and every 2 years thereafter (American Diabetes Association, 2000).

Overweight, defined as either: <ul style="list-style-type: none"> • BMI >85th percentile for age and sex • Weight for height >85th percentile • Weight >120% if ideal (50th percentile) for height
A family history of T2DM in first- or second-degree relatives
An at-risk ethnic group: <ul style="list-style-type: none"> • Native Americans • African-Americans • Hispanic-Americans • Asians • South Pacific islanders
Signs of insulin resistance or conditions associated with insulin resistance: <ul style="list-style-type: none"> • Acanthosis nigricans • Hypertension • Dyslipidemia • Polycystic ovarian syndrome

Table 1.1. Risk factors for type 2 diabetes in children and adolescents.

1.4 MONOGENIC DIABETES

A familial form of diabetes presenting during adolescence or in early adulthood (usually before the age of 25 years) was first described in the early 70's (Tattersall, 1974). Even though diabetes presented in young patients, the disease clinically resembled elderly-onset non-insulin dependent diabetes and the newly recognized subtype of familial diabetes became known by the acronym MODY (maturity-onset diabetes of the young). As MODY patients seemed to pass on the disease to their offspring following an autosomal dominant pattern, it was quickly suspected that it might be a monogenic disorder. However, the first causative genes were not identified until two decades later. MODY is both clinically and genetically heterogeneous. All currently known subtypes of MODY are caused by dominantly acting heterozygous mutations in genes important for the development or function of β -cells (Fajans et al, 2001).

In recent years, several forms of monogenic diabetes clinically different from MODY have been identified. Indeed, some of them show an autosomal recessive or X-linked recessive inheritance. In addition, patients with monogenic diabetes may have dominant mutations arising *de novo* (not inherited from parents), so there is no positive family history suggesting a monogenic condition. All these circumstances, along with the widespread lack of awareness, hinder clinical diagnosis. Since the vast majority of patients with genetically proven monogenic diabetes are initially incorrectly diagnosed as having type 1 or type 2 diabetes (Table 1.2), specific recommendations have been published to facilitate identification of patients with monogenic diabetes (Hattersley et al, 2009). These recommendations are based on the presence of certain clinical features or laboratory findings unusual in type 1 or type 2 diabetes (Table 1.3). None of these features is pathognomonic and therefore should be considered together rather than form individual (Hattersley et al, 2009).

	Type 1 diabetes	Type 2 diabetes	Monogenic diabetes
Genetics	Polygenic	Polygenic	Monogenic
Age at presentation	Throughout childhood (rare in infants)	Usually pubertal or postpubertal	Often pubertal or postpubertal (except GCK-MODY and neonatal diabetes)
Onset	Most often acute, rapid	Variable; from slow, mild (often insidious) to severe (DKA)	Variable
Associations:			
- Autoimmunity	Yes (>90%)	No	No
- Ketosis	Common	Rare	Rare in MODY, not uncommon in neonatal diabetes
- Obesity	Same risk as general population	Yes	Same risk as general population
- Acanthosis nigricans	No	Yes	No
Frequency (% of all diabetes in young people)	Usually >95%	<5-10% in most countries (60-80% in Japan)	1-3%
Diabetic parent	2-4%	80%	90%

DKA: Diabetic ketoacidosis

Table 1.2. Clinical characteristics of the different types of diabetes in children and adolescents (from Craig et al, 2009).

From a practical standpoint, monogenic diabetes can be categorized into five distinct groups; the first four subtypes are primarily due to β -cell failure (Murphy et al, 2008; Hattersley et al, 2009):

1. Neonatal diabetes and diabetes diagnosed in early infancy.
2. Autosomal dominant familial diabetes.
3. Familial, mild fasting hyperglycemia.
4. Diabetes mellitus associated with extrapancreatic features.
5. Monogenic insulin resistance syndromes.

Atypical features in type 1 diabetes:
<ol style="list-style-type: none"> 1. A diagnosis of diabetes before 6 months of age. 2. Family history of diabetes with a parent affected. 3. Evidence of endogenous insulin production outside the "honeymoon" phase (after 3-5 years of diabetes), with detectable C-peptide (>0.66 ng/mL or >200 nmol/L) when glucose level is (>145 mg/dL or >8 mmol/L). 4. Absent pancreatic islet autoantibodies, especially if measured at diagnosis. 5. Non-high risk HLA haplotype.
Atypical features in type 2 diabetes:
<ol style="list-style-type: none"> 1. Not markedly obese or diabetic family members who are of normal weight. 2. Absence of acanthosis nigricans. 3. Ethnic background with a low prevalence of type 2 diabetes (e.g., European Caucasian). 4. No evidence of insulin resistance with fasting insulin and/or C-peptide within the normal range.

Table 1.3. Features in children initially thought to have type 1 or type 2 diabetes that might suggest a monogenic subtype of diabetes (modified from Hattersley et al, 2009).

1.5 NEONATAL DIABETES AND DIABETES DIAGNOSED IN EARLY INFANCY

All cases of diabetes diagnosed before 6 months of age are likely to be monogenic and not classic autoimmune type 1 diabetes (Iafusco et al, 2002; Edghill et al, 2006). Neonatal diabetes is a rare and clinically heterogeneous disorder with an estimated prevalence of 1 case in 100,000 live births (Sperling, 2006). Many of these patients are born small for gestational age, which reflects a prenatal deficiency of insulin secretion as insulin exerts potent growth-promoting effects during intrauterine development (Gicquel et al, 2006). Diabetes resolves in approximately half of the patients (transient neonatal diabetes, TNDM), most often during the first year of life. The remaining patients continue to need treatment for life (permanent neonatal diabetes, PNDM) (von Muhlendahl et al, 1995). In both cases, diabetes can present isolated or associated with other clinical manifestations. At least 14 different loci for neonatal

diabetes have been described so far (Table 1.4). No clinical or laboratory features can help distinguish transient from permanent forms in patients with isolated neonatal diabetes. However, associated clinical features can be used to guide molecular testing when present (Hattersley et al, 2009).

Gene	Locus	Inheritance	Other clinical features
Abnormal pancreatic development:			
<i>ZAC/PLAGL1</i>	6q24	Variable (<i>imprinting</i>)	TNDM ± macroglossia
<i>ZFP57</i>	6p22.1	Recessive	TNDM + multisystemic problems
<i>IPF1</i>	13q12.1	Recessive	PNDM + pancreatic agenesis
<i>PTF1A</i>	10p12.3	Recessive	PNDM + pancreatic agenesis and cerebellar hypoplasia/aplasia
<i>HNF1B</i>	17cen-q21.3	Dominant	TNDM + pancreatic hypoplasia and renal cysts
<i>GLIS3</i>	9p24.3-p23	Recessive	PNDM + congenital hypothyroidism, hepatic fibrosis and renal cysts
Abnormal β-cell function:			
<i>KCNJ11</i>	11p15.1	Dominant	PNDM/ TNDM ± DEND
<i>ABCC8</i>	11p15.1	Dominant, Recessive	TNDM/PNDM ± DEND
<i>GCK</i>	7p15-p13	Recessive	Isolated PNDM
<i>SLC2A2</i> (GLUT2)	3q26.1-q26.3	Recessive	Fanconi-Bickel syndrome (hypergalactosemia and liver dysfunction)
<i>SLC19A2</i>	1q23.3	Recessive	Roger's syndrome (thiamine-responsive megaloblastic anemia, sensorineural deafness)
Destruction of β cells:			
<i>INS</i>	11p15.1	Dominant	Isolated PNDM
<i>EIF2AK3</i> (PERK)	2p12	Recessive	Wolcott-Rallison syndrome (skeletal dysplasia, recurrent liver dysfunction)
<i>FOXP3</i>	Xp11.23-p13.3	X-linked	IPEX syndrome (autoimmune enteropathy, eczema, autoimmune hypothyroidism, elevated IgE)

Table 1.4. Known genetic subtypes of neonatal diabetes (TNDM: transient neonatal diabetes; PNDM: permanent neonatal diabetes; DEND: developmental delay, epilepsy and neonatal diabetes; IPEX: immunodysregulation, polyendocrinopathy, enteropathy, X-linked).

1.5.1 Transient neonatal diabetes

A genetic diagnosis is now possible for most patients diagnosed with TNDM (Flanagan et al, 2007). The majority of cases (~70%) are linked to several abnormalities in an imprinted region on chromosome 6q24. Activating mutations in either of the genes encoding the two subunits of the ATP-sensitive potassium channel (K_{ATP}) of the β -cell membrane (*KCNJ11* or *ABCC8*) account for most of the remaining cases (~25%) (See below). Patients with 6q24 abnormalities show a more severe intrauterine growth retardation and are diagnosed earlier than patients with K_{ATP} channel mutations, indicating a more severe insulin deficiency during the last months of intrauterine development and at the time birth in the former. Furthermore, diabetes usually remits sooner and relapses later (Table 1.5).

Characteristic	6q24	<i>ABCC8</i>	<i>KCNJ11</i>	p value
Sex (% male)	53	54	58	NS
Gestational age (weeks)	40 (36-42)	39 (30-41)	38 (30-40)	NS
Birth weight (g)	1,950 (1,600-2,670)	2,575 (1,360-3,400)	2,570 (1,535-3,570)	<0.05
Age at diagnosis (weeks)	0 (0-4)	4 (0-9)	5 (0-16)	<0.05
Age at remission (weeks)	13 (5-60)	22 (7-52)	45 (2-208)	<0.05

Table 1.5. Clinical characteristics of probands with TNDM grouped by genetic etiology (adapted from Flanagan et al, 2007).

A. TNDM due to abnormalities on chromosome 6q24.

This is the single most frequent genetic cause of neonatal diabetes (Temple et al, 2000). The 6q24 region, where the candidate gene *ZAC* or *PLAGL1* maps to, is an imprinted region. Normally, the maternal allele is silenced and only the allele

inherited from the father is expressed. TNDM is ultimately associated with the overexpression of the gene (Ma et al, 2004). To date, three different molecular mechanisms leading to this overexpression have been reported: paternal uniparental disomy of chromosome 6 (either complete or partial; it accounts for 50% of sporadic TNDM cases), unbalanced paternal duplication of 6q24 (found in most familial cases), and abnormal methylation of the maternal allele (found in some sporadic cases) (Temple et al, 2002). Methylation defects may affect only the 6q24 locus or may arise in the context of a generalized hypomethylation syndrome along with other clinical features including congenital heart defects, brain malformations, etc. (Mackay et al, 2006). Some cases of TNDM secondary to multiple methylation defects are caused by recessively acting mutations in *ZFP57*, a gene on chromosome 6p involved in the regulation of DNA methylation (Mackay et al, 2008).

Patients with 6q24 abnormalities are born with intrauterine growth retardation (average weight: 1,930 g) and develop severe but non-ketotic hyperglycemia very early on, usually during the first week of life (Temple et al, 2002). Despite the severity of the initial presentation, the insulin dose can be tapered quickly so that the majority of patients do not require any treatment by a median age of 12 weeks. One third of patients show macroglossia and, more rarely, an umbilical hernia is present. During remission, transient hyperglycemia may occur during intercurrent illnesses (Shield et al, 2004). Over time diabetes relapses in at least 50-60% of patients, usually around puberty, although recurrences have been reported since age 4 years. Relapse clinically resembles early-onset type 2 diabetes and is characterized by a loss of the first-phase insulin secretion. Insulin therapy is not always necessary (there is usually some response to oral sulfonylureas) and, if needed, doses required tend to be lower than in patients with type 1 diabetes.

The phases described above do not present irremediably in every patient. Interestingly, some mutation carrier relatives develop type 2 diabetes or

gestational diabetes in adulthood without any evidences of having had neonatal diabetes, suggesting that other genetic or epigenetic factors may influence the clinical expression alterations of chromosome 6q24 (Temple et al, 2000).

Genetic counseling depends on the underlying molecular mechanism. Uniparental disomy of chromosome 6 is generally sporadic and therefore the risk of recurrence in siblings and offspring is low. When paternal duplication of the 6q24 region is found, males have a 50% chance of transmitting the mutation and the disease to their children. In contrast, females will pass on the duplication but their children will not develop the disease. In this case, TNDM may recur in the next generation as their asymptomatic sons pass on the molecular defect to their own children. Some methylation defects (*ZFP57* mutations) show an autosomal recessive inheritance and hence the recurrence risk is 25% for siblings and almost negligible for the offspring of a patient.

1.5.2 Permanent neonatal diabetes

In contrast to TNDM, the genetic basis of PNDM can be identified in about 60% of probands (Edghill et al, 2008). Most of them will have isolated PNDM as a result of mutations in one of four different genes (*KCNJ11*, *ABCC8*, *INS*, and *GCK*) (Figure 1.2). Only 10% of cases of diabetes is associated with pancreatic aplasia/hypoplasia or other extrapancreatic features (Table 1.4).

A. Neonatal diabetes due to activating mutations in the K_{ATP} channel.

The K_{ATP} channels are hetero-octameric complexes formed by four pore-forming Kir6.2 subunits and four SUR1 regulatory subunits, encoded by the genes *KCNJ11* and *ABCC8*, respectively (Miki et al, 2005). These channels regulate insulin secretion by linking intracellular metabolic state to the β -cell membrane electrical activity. In addition to pancreatic β -cells, they can be found in other cell

types, including neurons and muscle cells (Ashcroft, 2005). Any increase in the intracellular metabolic activity induces an increase in the ATP/ADP ratio within the cell, which makes the K_{ATP} channels close. This leads to the cell membrane depolarization that ultimately triggers insulin secretion (Ashcroft, 2005). Mutations in *KCNJ11* or *ABCC8* are found in nearly half of PNDM patients (~35% *KCNJ11*, ~13% *ABCC8*) (Flanagan et al, 2006; Ellard et al, 2007). These mutations prevent K_{ATP} channel closure, and hence insulin secretion, in response to hyperglycemia.

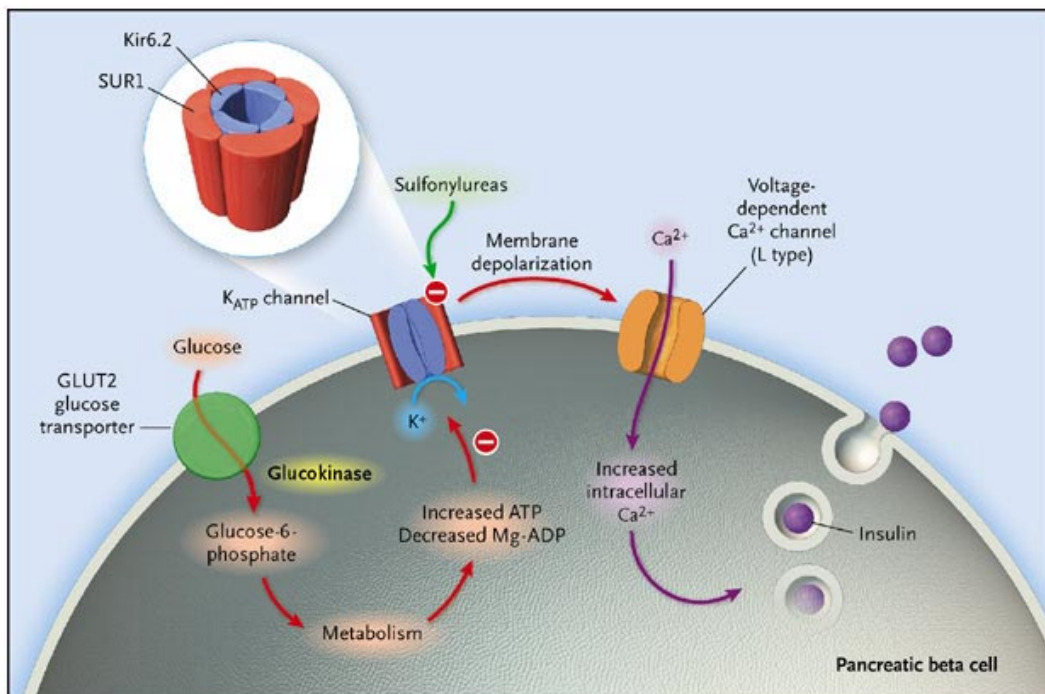


Figure 1.2. Schematic representation of the pancreatic β -cell, illustrating the role of glucokinase and the ATP-sensitive potassium (K_{ATP}) channel in insulin secretion. Glucose enters the β -cell by way of the GLUT2 glucose transporter. Once inside the cell, glucose is phosphorylated by glucokinase and metabolized, leading to changes in the intracellular concentration of adenine nucleotides that cause K_{ATP} channel closure. The K_{ATP} channel consists of four sulfonylurea-receptor (SUR1) subunits and four Kir6.2 subunits in an octomeric structure. Channel closure leads to membrane depolarization, which subsequently activates voltage-dependent calcium (Ca^{2+}) channels, leading in turn to an increase in intracellular Ca^{2+} , which triggers insulin exocytosis. Sulfonylureas initiate secretion by directly binding to the SUR1 subunits of K_{ATP} channels and causing channel closure (reproduced from Gloyn et al, 2004).

Mutations in either of the K_{ATP} channel genes can produce both TNDM and PNDM. Most patients with mutations in *KCNJ11* have isolated diabetes and the majority has PNDM (~90%) rather than TNDM (~10%). In contrast, mutations in *ABCC8* cause TNDM more frequently (~66%). There are no significant differences between the two subtypes of neonatal diabetes regarding the severity of intrauterine growth retardation or the age at diagnosis of diabetes (Tables 1.5 and 1.6). Approximately 30% of patients present in diabetic ketoacidosis. In keeping with the expression of K_{ATP} channels in brain and skeletal muscles, about 20% of probands with *KCNJ11* mutations present with associated neurological symptoms (Hattersley et al, 2005). The most severe defect includes marked developmental delay and early-onset epilepsy, and has been called DEND syndrome (Gloyn et al, 2006). An intermediate DEND syndrome characterized by neonatal diabetes and less severe developmental delay without epilepsy is more common. Neurological features are less frequent and usually milder (language delay and dyspraxia, mainly) in patients with mutations in *ABCC8* (Babenko et al, 2006; Ellard et al, 2007).

Characteristic	<i>KCNJ11</i>	<i>ABCC8</i>	<i>INS</i>	p value
Sex (% male)	53	42	50	NS
Gestational age (weeks)	40 (33-42)	40 (26-40)	40 (35-42)	NS
Birth weight (g)	2,660 (1,850-3,600)	2,700 (1,510-4,200)	2,700 (1,700-3,900)	NS
Age at diagnosis (weeks)	8 (0-33)	8 (0-40)	11 (0-1,144)	<0.05

Table 6. Clinical characteristics of the three commonest subtypes of PNDM (modified from Edghill et al, 2008).

Approximately 90% of patients with activating mutations in the K_{ATP} channel genes can be transferred from insulin onto sulfonylurea tablets (Pearson et al, 2006; Rafiq et al, 2008). Transfer usually improves glycemic control without increasing the risk of hypoglycemia. The required doses are typically higher than

those used in type 2 diabetes, although somewhat lower in patients with *ABCC8* mutations than in patients with mutations in *KCNJ11*. The only side effects reported to date are transient diarrhea and staining of the teeth (Codner et al, 2005; Kumaruguru et al, 2009). Preliminary data also suggest that glibenclamide can partially improve some of the neurological symptoms (Slingerland et al, 2006; Gurgel et al, 2007).

Activating mutations in *KCNJ11* causing neonatal diabetes are always heterozygous. Since about 90% of these mutations arise *de novo*, there is usually no family history of neonatal diabetes (Edghill et al, 2007). Familial cases show an autosomal dominant inheritance. Anyway, recurrence risk for the offspring is 50%. This is also true for most patients with activating mutations in *ABCC8*. However, some patients are homozygous or compound heterozygous for two different mutations and neonatal diabetes is recessively inherited (Ellard et al, 2007). In this case, the risk of neonatal diabetes for future siblings is 25% but almost inexistent for the offspring. Germline mosaicism (mutations present in the gonads but not detectable in blood) has been reported in several families (Gloyn et al, 2004; Edghill et al, 2007) and hence unaffected parents of a child with an apparently *de novo* mutation should be advised that the recurrence risk in siblings is low but not negligible.

B. PNDM due to mutations in the proinsulin gene.

Approximately 12% of patients with isolated PNDM have a heterozygous coding mutation in the preproinsulin gene (*INS*) (Støy et al, 2007; Edghill et al, 2008, Polak et al, 2008; Colombo et al, 2008). The mutation usually results in a misfolded proinsulin molecule that is trapped and accumulated in the endoplasmic reticulum, leading to endoplasmic reticulum stress and β -cell apoptosis (Eizirik et al, 2008).

The severity of intrauterine growth retardation in patients with *INS* mutations is similar to that of patients with K_{ATP} channel mutations. In contrast, diabetes presents at a slightly later age although the ranges overlap greatly (Table 6). Since there is a progressive β -cell death, insulin is the only treatment available.

The majority of *INS* mutations are sporadic *de novo* mutations. Only 20% of probands have a positive family history of autosomal dominant neonatal diabetes (Edghill et al, 2008).

Occasionally, *INS* mutations cause permanent diabetes after 6 months of age. Genetic testing should be considered in certain situations, especially in patients with antibody-negative type 1 diabetes (Polak et al, 2008; Molven et al, 2008).

C. PNDM due to mutations in the glucokinase gene.

The enzyme glucokinase is considered the glucose sensor of the β -cells, as it catalyzes the rate-limiting step of glucose phosphorylation and therefore enables the β -cell to respond appropriately to the degree of glycemia (Matschinsky, 2005).

Heterozygous mutations in the *GCK* gene produce familial mild non-progressive hyperglycemia (see below). However, complete glucokinase deficiency secondary to mutations in both alleles (either homozygous or compound heterozygous) prevents the β -cells from secreting insulin in response to hyperglycemia (Njølstad et al, 2001; Njølstad et al, 2003). For this reason, patients present with severe intrauterine growth retardation (mean birth: 1,700 g), are usually diagnosed with diabetes during the first few days of life, and require exogenous insulin therapy. Apart from diabetes, patients do not show any relevant extrapancreatic features.

GCK is responsible for not more than 2-3% of cases of PNDM. This type of PNDM is inherited in a recessive manner so the recurrence risk for future siblings

is 25%. This diagnosis should be strongly considered in probands born to consanguineous families, especially if both parents have mild hyperglycemia. However, the later is usually asymptomatic and measuring fasting blood glucose in the parents of any child with neonatal diabetes, even when there is no known family history of diabetes, is often recommended.

D. Other subtypes of PNDM.

The remaining known genetic causes of neonatal diabetes are rare (Table 1.3). Associated clinical features can be very helpful when planning genetic testing:

- Pancreatic aplasia or hypoplasia accounts for ~5% of PNDM cases. Most patients remain genetically undiagnosed, although two rare genetic causes have been identified in a few patients:
 - Complete deficiency of the transcription factor IPF1 secondary to homozygous or compound heterozygous mutations in the *IPF1* gene has been described in three probands with pancreatic agenesis (Stoffers et al, 1997; Schwitzgebel et al, 2003; Thomas et al, 2009). IPF1 is essential to embryonic development of the pancreas, as it regulates the differentiation process from mid-gut endodermic stem cells. In adults, it also participates in *INS* transcription. Thus, heterozygous mutations of *IPF1* are responsible for rare cases of familial early-onset diabetes (Stoffers et al, 1997).
 - Homozygous mutations in *PTF1A*, encoding the pancreas transcription factor 1- α , have been identified in three patients with pancreatic and cerebellar hypoplasia/agenesis from two unrelated consanguineous families (Hoveyda et al, 1999; Sellick et al, 2004). All of them died during infancy due to central respiratory failure.

- *GLIS3* is a widely expressed transcriptional regulator that has recently been shown to be involved in the production of a complex syndrome including neonatal diabetes, congenital hypothyroidism and dysmorphic features (Taha et al, 2003; Senee et al, 2006). Some patients also presented with congenital glaucoma, hepatic fibrosis and renal cysts. To date, homozygous mutations in the *GLIS3* gene have been reported in 4 probands from 3 unrelated consanguineous families.
- IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked) syndrome is a multisystemic disorder that presents in hemizygous males with a mutation in the *FOXP3* gene (Wildin et al, 2002). The protein encoded by this gene is necessary for the proper development and function of regulatory T cells (Ochs et al, 2007). Its lack is associated with the presence of numerous early-onset autoimmune diseases (enteropathy, diabetes, eczematous dermatitis, hypothyroidism, cytopenias, etc.) that leads to the patient's death usually within the first years of life. Remarkably, antibodies against β -cell antigens can be found, representing an important difference with other causes of PNDM. Immunosuppressive drugs and bone marrow transplantation are both included in the treatment regimen. Heterozygous carrier females remain asymptomatic.

The remaining subtypes of diabetes associating extrapancreatic features, although having been described in a number of patients with PNDM, produce diabetes mainly beyond the first 6 months of life and hence will be described later.

1.6 FAMILIAL DIABETES WITH AUTOSOMAL DOMINANT INHERITANCE

The sometimes confusing term MODY originates from the time when the terms juvenile-onset and maturity-onset were used to distinguish between type 1 (insulin-dependent) and type 2 (non insulin-dependent) diabetes. MODY was used to describe a subgroup of autosomal-dominantly inherited diabetes that was initially non insulin-dependent despite having a young age of onset (at least one family member diagnosed before 25 years of age). The different genetic subtypes differ in age of onset, pattern of hyperglycemia, response to treatment and associated extrapancreatic manifestations, which suggests that it is inappropriate to place them into a single category.

Four of the 6 classic MODY subtypes (MODY1, 3, 4 and 6) are characterized by familial symptomatic diabetes with no extrapancreatic features. All are caused by heterozygous mutations in a gene encoding a β -cell transcription factor (*HNF4A*, *HNF1A*, *IPF1*, and *NEUROD1*, respectively) and show an autosomal dominant inheritance (Stride et al, 2002).

1.6.1 *HNF1A* (MODY3) and *HNF4A* (MODY1) diabetes

These two subtypes of monogenic diabetes are by far the most common forms of monogenic diabetes secondary to a mutation in a transcription factor, although most cases are misdiagnosed as type 1 or type 2 and remain unidentified. *HNF1A* mutations are about 10 times more frequent than mutations in *HNF4A* (Pearson et al, 2005). Therefore, *HNF1A* is the first diagnostic possibility to be considered in families with autosomal dominant diabetes.

The glucose intolerance usually becomes evident during adolescence or early adulthood. In the early stages of the disease, fasting blood glucose may be normal but

patients tend to show a large increment in blood glucose (>80 mg/dL or 5 mmol/L) after meals or at 2 hours during an oral glucose tolerance test (OGTT) (Stride et al, 2002). Over time, fasting hyperglycemia and osmotic symptoms (polyuria, polydipsia) present but patients rarely develop ketosis because some residual insulin secretion persists for many years. Chronic complications of diabetes are frequent and their development is related to the degree of metabolic control (Isomaa et al, 1998). The frequency of microvascular complications (retinopathy, nephropathy, neuropathy) is similar to that of patients with type 1 and type 2 diabetes. However, *HNF1A* mutations are associated with an increased frequency of cardiovascular disease, although patients usually have plasma levels of HDL cholesterol higher levels that do not, however, seem to be cardioprotective (Pearson et al, 2003; Hattersley, personal communication).

Mutations in *HNF1A* show a high penetrance so that 63% of mutation carriers develop diabetes before 25 years of age, 79% before age 35 and 96% before 55 years (Murphy et al, 2008). The age at diagnosis of diabetes is partly determined by the location of the mutation within the gene (Harries et al, 2006; Bellanné-Chantelot et al, 2008). Patients with mutations affecting the terminal exons (8-10) are diagnosed, on average, 8 years later than those with mutations in exons 1 to 6. On the other hand, exposure to maternal diabetes *in utero* (when the mutation is maternally inherited) brings forward the age at onset of diabetes by about 12 years (Stride et al, 2002). Diabetes in *HNF4A* mutation carriers tend to appear slightly later than in patients with mutations in *HNF1A* (Hattersley, personal communication).

There are some differential clinical characteristics between patients with mutations in *HNF4A* and *HNF1A* that can help decide which gene should be considered first in a particular family:

- Patients with *HNF1A* mutations typically have a low renal threshold for glucose reabsorption due to impaired renal tubular transport of glucose. Slightly over half of children and adolescents carrying a mutation have postprandial glycosuria before developing significant hyperglycemia (Stride et

al, 2005). A positive urine test for glycosuria after a large unrefined carbohydrate meal could, therefore, suggest the need for predictive genetic testing in asymptomatic young children from families with *HNF1A* diabetes.

- Mutations in *HNF4A* are associated with relatively low levels of HDL cholesterol and high levels of LDL cholesterol. However, the most relevant clinical feature is that about 50% of *HNF4A* mutation carriers are macrosomic at birth (weight >4400 g) and 15% have diazoxide-responsive neonatal hyperinsulinemic hypoglycemia (Pearson et al, 2007). In this case, hyperinsulinism usually remits during infancy and patients develop diabetes from adolescence (Kapoor et al, 2008).

Patients with both *HNF1A* and *HNF4A*-diabetes are extremely sensitive to sulfonylureas (Pearson et al, 2003) and the glycemic control is usually better than that achieved on insulin, especially in children and young adults (Byrne et al, 1996). The initial dose should be low (one-quarter of the normal starting dose in adults) to avoid hypoglycemia (Hattersley et al, 2009). As long as the patients do not have problems with hypoglycemia, they can be maintained on low-dose sulfonylureas (e.g. 20–40 mg gliclazide daily) for decades. If there is hypoglycemia despite dose titration of a once or twice daily sulfonylurea preparation, a slow release preparation or meal time doses with a short-acting agent like nateglinide may be considered (Tuomi et al, 2006).

1.6.2 Other subtypes

Dominantly acting mutations in other transcription factors [*IPF1* - *MODY4* (Stoffers et al, 1997), *NEUROD1* - *MODY6* (Malecki et al, 1999), *KLF11* (Neve et al, 2005), *PAX4* (Plengvidhya et al, 2007) and *ISL1* (Shimomura et al, 2000)] have been reported in a handful of pedigrees with familial diabetes. They are extremely rare and therefore they are not usually considered in children and adolescents with diabetes in a clinical setting.

1.7 FAMILIAL MILD FASTING HYPERGLYCEMIA

The incidental finding of mild hyperglycemia (100-145 mg/dL or 5.5-8 mmol/L) in asymptomatic children and adolescents raises the possibility that these patients subsequently develop type 1 diabetes. In the absence of pancreatic autoimmunity markers, that risk is minimal (Lorini et al, 2001). However, a significant proportion will have a heterozygous mutation in the *GCK* gene (MODY2) (Feigerlová et al, 2006; Lorini et al, 2009). This is the commonest subtype of monogenic diabetes in the pediatric diabetes clinic.

The phenotype associated with heterozygous *GCK* mutations is remarkably homogeneous. In contrast to other subtypes of monogenic diabetes, *GCK*-MODY patients regulate insulin secretion adequately but around a slightly higher set point than normal controls. As a result, they show non-progressive mild hyperglycemia from birth. Their hemoglobin A1c is mildly elevated but usually below 7.5%. Despite the mild fasting hyperglycemia, there is a small increment in blood glucose during an OGTT (<60 mg/dL or <3.5 mmol/L) (Stride et al, 2002). Since the degree of hyperglycemia is not high enough to cause osmotic symptoms, most cases are usually diagnosed incidentally when blood glucose is measured for any other reason. Very often, the affected parent remains undiagnosed or has been misdiagnosed with early-onset type 2 diabetes.

Since blood glucose does not deteriorate significantly over time, this subtype of monogenic diabetes is rarely associated with chronic complications of diabetes (Velho et al, 1997) and patients do not generally require any treatment. Of note, the presence of a *GCK* mutation does not protect against the concurrent development of polygenic type 2 diabetes later in life, which occurs at a similar prevalence than in the general population (Murphy et al, 2008).

1.8 MULTISYSTEMIC GENETIC SYNDROMES ASSOCIATED WITH DIABETES

A monogenic disorder should be considered in any child with diabetes associated with a number of extrapancreatic features. Some of these syndromes cause neonatal diabetes and have been described above (Table 1.4). The most common ones causing diabetes beyond the first 6 months of life are listed below.

1.8.1 Renal cysts and diabetes (RCAD) syndrome (MODY5)

HNF1B is a transcription factor expressed during early embryonic development in the kidney, pancreas, liver and genital tract. Heterozygous mutations in *HNF1B* are less common than other forms of MODY. Patients rarely present with isolated diabetes (Bingham et al, 2004). In contrast, genetic testing should be considered in patients with developmental renal abnormalities, even in the absence of diabetes (Ulinski et al, 2006).

The renal involvement is characterized mainly by renal cysts and renal dysplasia although a number of other presentations are possible (Edghill et al, 2006). Genital-tract malformations (particularly uterine abnormalities), hyperuricemia and gout can also occur, as well as abnormal liver function tests (Bingham et al, 2004). Approximately half of *HNF1B* mutation carriers will develop diabetes during adolescence or early adulthood. They are insulin deficient but also have some degree of hepatic insulin resistance (Pearson et al, 2004). Furthermore, *HNF1B* mutations have also been reported in three probands with transient neonatal diabetes (Yorifuji et al, 2004; Edghill et al, 2006; Haumaitre et al, 2006). The coexisting pancreatic hypoplasia (Bellanné-Chantelot et al, 2004) and hepatic insulin resistance explain why patients with *HNF1B* diabetes do not respond adequately to sulfonylurea treatment and early insulin therapy is required (Murphy et al, 2008).

The phenotype of RCAD patients is highly variable even within families sharing the same *HNF1B* mutation. Therefore, this disorder should be considered not only in the diabetes clinic but also in other clinics (nephrology, urology and gynecology, at least). Importantly, a family history of renal disease or diabetes is not essential to prompt a screen for this disorder, as spontaneous mutations and deletions of this gene are common (one-third to two-thirds of cases) (Bellanné-Chantelot et al, 2005; Ulinski et al, 2006).

1.8.2 Wolfram syndrome (Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy and Deafness, DIDMOAD)

The association of diabetes with progressive optic atrophy below 16 years of age is diagnostic of this autosomal recessive syndrome (Domenech et al, 2006). Insulin-deficient diabetes is usually the first manifestation of the disease and presents at a mean age of 6 years. Over time, other features such as sensorineural deafness, central diabetes insipidus, urinary tract dilatations and neurological symptoms develop. These can present in a variable order, even within the same family. Patients with Wolfram syndrome die at a median age of 30 years, mainly from neurodegenerative complications.

At least 90% of patients harbors recessively-acting mutations in the *WFS1* gene (Khanim et al, 2001). A second variant of the syndrome has recently been described in association with mutations in *CISD2* (Amr et al, 2007). Patients with this rare variant do not develop diabetes insipidus but present with additional symptoms including bleeding diathesis and peptic ulcer disease.

1.8.3 Mitochondrial diabetes (maternally inherited diabetes and deafness, MIDD)

The most common form of mitochondrial diabetes is caused by a m.3243A>G mutation in mitochondrial DNA. Since all mitochondria in an individual come from the

mother, only females transmit the disease to their offspring (Murphy et al, 2008). Diabetes onset is usually insidious but approximately 20% of patients have an acute presentation, even in diabetic ketoacidosis (Maassen et al, 2004). Although it typically presents in adulthood, some cases have been reported in adolescents with a high degree of heteroplasmy (Guillausseau et al, 2004). Mitochondrial diabetes should be suspected in all patients presenting with diabetes mellitus and sensorineural hearing loss inherited from mother's side. Interestingly, the same m.3243A>G mutation also causes a much more severe clinical syndrome known as MELAS (myopathy, encephalopathy, lactic acidosis and stroke) (Goto et al, 1990).

Patients with mitochondrial diabetes may respond initially to diet or oral hypoglycemic agents but often require insulin treatment within months or years. Metformin should be avoided as it interferes with mitochondrial function and may trigger episodes of lactic acidosis.

The penetrance of diabetes in mutation carriers depends on the age considered, but is estimated to be above 85% at 70 years (Maassen et al, 2004). Affected males do not transmit the disease to their offspring. In contrast, females transmit the mutation to all her children, although some may not develop the disease (Murphy et al, 2008).

Besides the m.3243A>G mutation, early-onset diabetes (even in infancy) has been reported in other less common mitochondrial diseases such as Kearns-Sayre syndrome and Pearson syndrome (Laloi-Michelin et al, 2006; Superti-Furga et al, 1993).

1.8.4 Wolcott-Rallison syndrome

Biallelic mutations in *EIF2AK3* cause this rare autosomal recessive syndrome characterized by early-onset diabetes mellitus, spondyloepiphyseal dysplasia, and recurrent hepatic and/or renal dysfunction (Delepine et al, 2000; Senee et al, 2004). *EIF2AK3* (eukaryotic translation initiation factor alpha 2-kinase 3) encodes a protein

involved in the regulation of the endoplasmic reticulum stress response. This occurs when there is an imbalance between protein synthesis and processing so that misfolded proteins accumulate in the endoplasmic reticulum. Pancreatic development is rather normal in the absence of the functional protein but misfolded proteins accumulate within the endoplasmic reticulum after birth and eventually induce β -cell apoptosis. Diabetes usually manifests during infancy, but may do so within the first 3-4 years of age.

1.8.5 Roger's syndrome

Thiamine-responsive megaloblastic anemia (TRMA) is an autosomal recessive disorder characterized by diabetes, megaloblastic anemia, and sensorineural deafness. Some patients also present with congenital heart disease, optic atrophy, retinal degeneration and stroke. The disease is caused by biallelic mutations in *SLC19A2* (Labay et al, 1999). This gene encodes a widely expressed cell membrane thiamine transporter known as THTR1. Insulin-deficient diabetes, like most of the associated clinical features, initially responds to the administration of thiamine supplements. However, the majority of patients need insulin after puberty (Ricketts et al, 2006). Although the disease usually presents in toddlers and preschoolers, some cases have been reported from 3 months of age (Mandel et al, 1984).

1.8.6 Fanconi-Bickel syndrome

Mutations in the *SLC2A2* gene, which encodes the glucose transporter of the plasma membrane of β -cells (GLUT2), are responsible for a rare autosomal recessive syndrome characterized by abnormal metabolism of glucose and galactose, accumulation of glycogen in the liver and kidneys, and renal proximal tubular dysfunction (Santer et al, 1998). Usually patients show fasting hypoglycemia and postprandial hyperglycemia, but a

number of cases with neonatal diabetes have been described (Kentrup et al, 1999; Yoo et al, 2002).

1.8.7 Diabetes secondary to monogenic diseases of the exocrine pancreas

Heterozygous mutations in *CEL*, which encodes a pancreatic lipase, cause an autosomal dominant disorder of pancreatic exocrine insufficiency and diabetes (Raeder et al, 2006). Other autosomal dominant monogenic diseases affecting mainly the exocrine pancreas that can lead to diabetes sooner or later include cystic fibrosis (*CFTR*) (Moran et al, 2003) and hereditary pancreatitis (*PRSS1* and *SPINK1*) (Teich et al, 2008).

1.9 MONOGENIC INSULIN RESISTANCE SYNDROMES

Monogenic diabetes associated with extreme insulin resistance is less common than monogenic β -cell failure.

1.9.1 Insulin receptor (*INSR*) gene mutations

Biallelic *INSR* mutations cause a number of very rare recessive syndromes characterized by severe insulin resistance and greatly increased insulin and C-peptide levels. There is a strong genotype-phenotype correlation (Longo et al, 2002; Musso et al, 2004):

- Nonsense mutations or mutations in the extracellular domain of the receptor produce the most severe syndrome, referred to as *Donohue syndrome* or *leprechaunism*. It is characterized by intrauterine growth retardation,

abnormal glucose metabolism and dysmorphic features (low-set ears, thick lips, flat nose root, thick skin, lack of subcutaneous fat, hypertrichosis and macrogenitalism). Most patients die during infancy.

- Less severe mutations in the insulin-binding domain or in the intracellular domain of the receptor lead to *Rabson–Mendenhall syndrome*. Affected patients present with dysplastic teeth and gums, thickened nails and hirsutism. Recently, an association with medullary sponge kidney has been described. Most patients die before adolescence.

In both cases, patients initially have fasting hypoglycemia and postprandial hyperglycemia, but eventually develop persistent hyperglycemia and ketoacidosis.

- Type A insulin resistance syndrome is characterized by severe insulin resistance in the absence of obesity. It is usually diagnosed in non-obese female adolescents with severe acanthosis nigricans and hyperandrogenism (polycystic ovarian syndrome). Abnormal glucose tolerance develops later on.

Treatment of severe insulin resistance is mostly ineffective. Metabolic control remains poor and long-term diabetes complications are frequent. Approaches used include the use of the insulin sensitizers, i.e. metformin and/or glitazones, but their impact is limited when the insulin resistance is very severe. Insulin is the main stay of treatment, but extraordinarily high doses are needed, and 500U insulin and insulin pumps are usually required (Hattersley et al, 2009). As an alternative therapeutic method, recombinant human insulin-like growth factor-I (rhIGF-I) has been reported to be successful in treatment of Donohue syndrome and type A insulin resistance (Kuzuya et al, 1993). This treatment is effective in lowering both fasting and postprandial plasma glucose concentrations, and in some cases, improvement of acanthosis nigricans was also observed; however, the long-term effect is still unsatisfactory (Nakae et al, 1998).

1.9.2 Genetic lipodystrophies

Lipodystrophies are characterized by a selective lack of adipose tissue. This results in a decreased adipokines levels and insulin resistance (Garg, 2004).

- *Congenital generalized lipodystrophy (Berardinelli–Seip syndrome)* is a recessive disorder characterized by an almost complete absence of subcutaneous and visceral fat with severe hyperinsulinemia that progresses to diabetes in early adolescence. Patients may show acanthosis nigricans, extreme hypertriglyceridemia, fatty liver, virilisation and cardiomyopathy. Mutations in two genes (*AGPAT2* and *BSCL*) account for approximately 80% of cases (Agarwal et al, 2003). Extremely high doses of insulin are needed for metabolic control. In contrast, the response of diabetes to recombinant leptin can be dramatic but it is only available on a research basis (Beltrand et al, 2007).
- Patients with familial partial lipodystrophy show loss of subcutaneous fat from the extremities, lower trunk and the gluteal region. They exhibit a severe insulin resistance phenotype with hyperinsulinemia, hypertriglyceridemia and decreased HDL cholesterol. Diabetes usually appears in late adolescence or early adulthood. Dominantly acting heterozygous mutations in *LMNA* or *PPARG* account for approximately 50% of cases (Garg, 2004), which suggests that additional loci may exist. Mutations in *ZMPSTE24*, *AKT2*, and *CIDEA* have been identified in a few cases (Garg et al, 2009; Rubio-Cabezas et al, 2009), but many patients still remain undiagnosed. Insulin treatment together with metformin is still not sufficient in most cases. Due to their PPAR γ agonist effect, thiazolidinediones may be of benefit (Arioglu et al, 2000).

1.9.3 Alström syndrome

This autosomal recessive disorder shares symptoms with Bardet-Biedl syndrome (See below), including retinitis pigmentosa, deafness, obesity, and diabetes mellitus. It can be distinguished from the latter syndrome by the lack of polydactyly and hypogonadism and by the absence of mental impairment (Alström et al, 1959). The syndrome is caused by mutations within the *ALMS1* gene of unknown function (Hearn et al, 2002). Patients with Alström syndrome show many features of the metabolic syndrome including hyperlipidemia, hyperuricemia, insulin resistance, hypertension, and diabetes. Furthermore, acanthosis nigricans, chronic active hepatitis (possibly based on non-alcoholic steatohepatitis) and dilated cardiomyopathy have also been reported.

1.9.4 Bardet-Biedl syndrome

This (in most cases) autosomal recessive disorder is characterized by mental retardation, pigmentary retinopathy, polydactyly, obesity, diabetes mellitus, renal dysplasia, hepatic fibrosis, and hypogonadism (Beales et al, 1999). Obesity is found in almost every patient, while diabetes affects less than 50%. While the syndrome shares some similarities with Lawrence-Moon syndrome, these two disorders can be distinguished by the presence of paraplegia and the absence of polydactyly, obesity, and diabetes mellitus in Lawrence-Moon syndrome. Terms such as Lawrence-Moon-Bardet-Biedl or Lawrence-Moon-Biedl syndrome should therefore be avoided. Bardet-Biedl syndrome has been linked to at least 14 different genetic loci, referred to as BBS1 to BBS14 (Stoetzel et al, 2007; Leitch et al, 2008). Heterozygous carriers possibly exhibit an increased risk for obesity, hypertension, diabetes mellitus, and renal disease.

1.10 RATIONALE

The vast majority of diabetic children have type 1 diabetes, while monogenic diabetes is responsible for only 1-2% of cases. Despite the recent advances in the understanding of the molecular basis of monogenic diabetes outlined above, the translation of the new knowledge into changes in daily clinical care usually occurs very slowly. As a consequence, many pediatric patients with monogenic diabetes continue to be initially misdiagnosed as having type 1 diabetes, especially where pancreatic antibody measurement is not routinely performed in the clinical setting.

Whilst genetic testing confirms or excludes a diagnosis of monogenic diabetes with both high sensitivity and high specificity, molecular studies are expensive and some criteria must be used to select candidates to genetic testing in order to approach the problem in a cost-effective way.

Age at diabetes onset can be considered one of these cornerstone criteria. Six months is a reasonable cut-off as classic polygenic autoimmune type 1 diabetes will account for most cases presenting during late infancy (6 to 12 months), childhood or adolescence, whereas most infants diagnosed before 6 months will have monogenic diabetes. However, some patients diagnosed after 6 months will also have monogenic diabetes. Therefore, a different approach for selecting candidates for genetic testing must be used. Since the number of genes associated with neonatal and early infancy onset diabetes is relatively high, additional selection criteria are also needed in this case.

The history and physical examination are the best and cheapest tools available for this purpose (Barrett, 2007). Specific clinical features associated with a certain genetic syndrome can be easily identified and help guide the genetic testing. The existence of consanguinity or a history of miscarriages or infant mortality suggests a possible autosomal recessive syndrome. A positive family history of diabetes is an important clue, especially if the disease follows an autosomal dominant inheritance pattern (familial

diabetes or MODY) or is associated with deafness or epilepsy only on the maternal side (mitochondrial diabetes).

Some laboratory tests may also prove useful, especially the measurement of autoantibodies to β -cells and plasma levels of insulin and/or C-peptide. In selected patients, other tests should be considered including, but not limited to, liver and kidney function tests, X-ray bone survey, audiogram, echocardiogram and/or brain MRI.

In order to identify children with monogenic diabetes accurately, it is therefore important to define the most informative clinical and laboratory findings pointing at a specific gene. Although pancreatic autoantibodies are considered the diagnostic hallmark for type 1 diabetes, their usefulness (when absent) as a selection tool for genetic testing in patients with isolated diabetes needs to be further explored.

New monogenic syndromes are usually described in affected patients showing a severe phenotype. However, as soon as the causative gene is identified, it is not uncommon that less severe mutations are reported in patients with milder oligosymptomatic forms of the disorder. Whether this is also true for some subtypes of monogenic diabetes remains to be determined. In addition, a genetic cause can be identified in only 60% of PNDM patients indicating there must be other genes to be identified. Some of these patients have isolated diabetes whereas others present with complex multisystemic syndromes. Considering neonatal diabetes as a developmental disorder, genes encoding transcription factors central to pancreatic or β -cell development are especially interesting as potential novel genetic causes.

1.11 OBJECTIVES

The aims of the present work were:

- I. To explore the contribution of common causes of monogenic diabetes to the production of idiopathic type 1 diabetes in children.
- II. To investigate the frequency of autoimmune diabetes among patients with permanent neonatal diabetes.
- III. To increase the current knowledge on the molecular bases of monogenic diabetes in newborns and infants.

1.12 OUTLINE OF THE THESIS

This doctoral dissertation gives a detailed account of five different studies. **Chapter 2** describes the genetic screening of four relatively common causes of monogenic diabetes (*HNF1A*, *HNF4A*, *KCNJ11* and *INS*) in children clinically diagnosed as having antibody-negative type 1 diabetes. **Chapter 3** describes the relative frequency of monogenic autoimmune diabetes secondary to *FOXP3* mutations among male patients with PNDM from a large international cohort. In addition to the well-known IPEX syndrome, a milder phenotype is reported for the first time. **Chapter 4** describes in detailed a patient with a homozygous nonsense mutation in *GCK*. **Chapter 5** explores the contribution of *EIF2AK3* mutations to PNDM and the identification of potential candidates for genetic testing before the full clinical picture develops by using a novel approach based on homozygosity mapping. **Chapter 6** reports the identification of recessively acting mutations in the *INS* gene, encoding proinsulin, as a novel cause of neonatal diabetes. During this study, a previously uncharacterized region in the *INS* promoter has been identified. **Chapter 7** describes a novel genetic subtype of PNDM due to

homozygous mutations in *NEUROD1*, a gene central to pancreatic islet development and β -cell function. **Chapter 8** discusses the significance of the presented data in the context of the literature, as well as some recommendations for future research. Finally, **Chapter 9** summarizes this dissertation and gives the final conclusions, both in English and Spanish.

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CHAPTER 2

Testing for monogenic diabetes among children and adolescents with antibody-negative clinically defined type 1 diabetes

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ABSTRACT

Aims: Monogenic diabetes is frequently misdiagnosed as type 1 diabetes. We aimed to screen for undiagnosed monogenic diabetes in a cohort of children who had a clinical diagnosis of type 1 diabetes but were pancreatic autoantibody-negative.

Methods: We studied 252 patients clinically diagnosed with type 1 diabetes between 6 months and 17 years old. Pancreatic autoantibodies (ICA, GADA and/or IA2A) were absent in 25 cases (9.9%). The most frequent genes involved in monogenic diabetes [*KCNJ11* and *INS* for neonatal diabetes and *HNF1A* and *HNF4A* for maturity-onset diabetes of the young (MODY)] were directly sequenced.

Results: Two of the twenty five (8%) antibody-negative patients had de novo heterozygous mutations in *INS*; c.94G>A (G32S) and c.265C>T (R89C). The two patients presented with non-ketotic hyperglycaemia at 8 and 11 months. In contrast, the four antibody-positive patients who presented at a similar age (6-12 months) showed a more severe metabolic derangement, manifested as ketosis in all four cases, with ketoacidosis in two. At age 15 and 5 years both *INS* mutation patients were prescribed a replacement dose of insulin with good glycemic control [glycated hemoglobin (HbA1c) 7.0 and 7.2%]. No mutations were found in *KCNJ11*, *HNF1A* or *HNF4A*.

Conclusions: The identification of patients with monogenic diabetes from children with clinically defined type 1 diabetes may be helped by clinical criteria including the absence of pancreatic autoantibodies.

INTRODUCTION

Immune-mediated destruction of the pancreatic β -cells underlies the commonest subtype of diabetes mellitus in children, type 1 diabetes (1). Although destruction of β -cells is considered T-cell mediated, anti-islet autoantibodies are the best characterized markers of β -cell autoimmunity. However, some patients do not present with autoimmune markers and are classified as having antibody-negative type 1 diabetes, also referred to as idiopathic or type 1b diabetes (1). Monogenic diabetes is rare in children and is often misdiagnosed as type 1 diabetes (2-7). We hypothesized that it could explain some cases of apparent type 1 diabetes in children. The aim of the present study was therefore to search for a monogenic cause in selected children with antibody-negative type 1 diabetes.

PATIENTS AND METHODS

We retrospectively reviewed the medical records of 280 patients with clinically-diagnosed type 1 diabetes actively followed in the paediatric diabetes clinic at the Hospital Infantil Universitario Niño Jesús, Madrid, Spain. Median age at onset of diabetes was 5.9 years [interquartile range (IQR): 3.1 – 9.0], ranging from 0.7 to 16.4 years.

Information on islet cell antibodies (ICA) within one week of diagnosis was available for 224 cases. The presence of ICA was investigated by immunofluorescence using human pancreatic tissue and titres ≥ 5 Juvenile Diabetes Foundation (JDF) units were considered to be positive. There were no differences in clinical characteristics between patients who had or had not been tested for ICA (data not shown). The 56 patients without an ICA result and the 47 patients with a negative ICA result ($n=103$) were invited for additional antibody studies and, if these were negative, genetic testing was performed. Twenty-eight patients declined to take part in further testing. Thus, we are only able to give results on 252 subjects.

A random venous blood sample was taken and glutamic acid decarboxylase isoform 65 and insulinoma-associated antigen-2 antibodies (GADA and IA2A, respectively) were measured by ELISA (DRG Instruments GmbH, Marburg, Germany), at a median 5.3 years after diabetes onset (IQR: 3.2-9.2). The cut-offs to define antibody status as negative were GADA levels <10 U/mL and IA2A levels <20 U/mL.

Patients with either positive ICA at diagnosis or positive GADA and/or IA2A during follow-up were considered to have autoimmune type 1 diabetes (n = 227). The remaining 25 patients were classified as having antibody-negative type 1 diabetes, nine of whom had a negative ICA result at diabetes onset.

Genetic testing was performed on the antibody negative subjects. Genomic DNA was extracted from peripheral leukocytes using standard procedures. The coding exons and intron-exon boundaries of *KCNJ11*, *INS*, *HNF1A* and *HNF4A* were amplified by PCR (primers and conditions available upon request). Single-strand sequencing was carried out using standard methods on an ABI 3730 sequencer (Applied Biosystems, Warrington, UK). Sequences were compared with the published reference templates (NM_000525, NM_000207, NM_000545.4 and NM_000457.3, respectively) using Mutation Surveyor v3.20 (SoftGenetics, State College, PA, USA). Changes in the sequence were checked against published polymorphisms and mutations. Gene deletions in the aforementioned genes and other rare causes of monogenic diabetes, including *ABCC8* and *HNF1B* mutations, have not been addressed in this study. As all the patients presented with symptomatic random hyperglycaemia and HbA1c >7.5%, *GCK* mutations were not considered likely and they have not been tested.

Written informed consent was obtained from all participants or their parents. The study was approved by the local Ethics Committee and performed according to the Helsinki Declaration, as revised in 2000.

RESULTS

The 25 (9.9%) patients with antibody-negative diabetes were diagnosed at a younger age than the 227 patients with diabetes and pancreatic autoantibodies [median 3.85 (IQR: 1.74–6.45) vs. 6.29 (IQR: 3.16–9.43) years, $p=0.01$]. GADA and IA2A were tested significantly later after diabetes onset in patients with a negative result [median 7.60 (IQR: 4.02–11.58) vs. 4.95 (IQR: 2.50–7.90) years, $p=0.02$].

Two of the twenty five antibody negative patients (8%) had monogenic diabetes. These two patients had heterozygous mutations in *INS*; c.94G>A (G32S) and c.265C> T (R89C). These mutations have been previously reported as being pathogenic (8). They were not found in unaffected parents, representing *de novo* mutations, and were not seen in 222 UK Caucasian control chromosomes. No mutations were found in *KCNJ11*, *HNF1A* or *HNF4A*.

The patient with the G32S mutation presented with progressive polyuria and polydipsia at 11 months of age. Blood tests revealed hyperglycaemia without ketonuria and a normal C-peptide level (929 pmol/L) that was still measurable one year after diabetes onset (570 pmol/L). She is currently 15 years old and has good metabolic control (HbA1c: 7.0%) on a full replacement insulin dose (0.92 U/kg/day).

The proband with the R89C mutation presented with a persistent nappy rash at 8 months. Following further investigation, glycosuria was found and the parents recalled some degree of polyuria and polydipsia during the previous weeks. The child was hyperglycaemic but not ketotic. At 5 years of age, he has acceptable metabolic control (HbA1c: 7.2%) on a basal-bolus insulin regimen (0.48 U/kg/day).

There were four other children in this cohort who were diagnosed before 1 year of age but all of them were considered to have autoimmune type 1 diabetes (Table 1). The youngest case was diagnosed at the age of 10 months. In contrast to the *INS* mutation carriers, they presented with either ketosis or ketoacidosis. Birth weight did not discriminate the two patients with an *INS* mutation from the remainder of the group.

Type of diabetes	Gender	Clinical data at diabetes onset						Clinical data at birth			
		Age (moths)	Osmotic symptoms	Glucose (mmol/L)	Ketosis	Venous pH	Bicarbonate (mmol/L)	Gestational age (wks)	BW [#] (g)	BW [#] (SDS)	BW [#] (centile)
INS mutation (R89C)	Male	8.1	Yes	24.7	No	7.30	21.8	42	3400	-0.80	21
INS mutation (G32S)	Female	11.2	Yes	16.2	No	7.37	19.0	39	3250	-0.20	42
Type 1 diabetes (ICA: 20 JDF)	Male	10.3	Yes	36.9	Yes	7.03	6.0	40	3450	-0.21	42
Type 1 diabetes (ICA: 80 JDF)	Male	10.4	Yes	22.6	Yes	7.35	17.4	40	3980	0.86	81
Type 1 diabetes (ICA: 160 JDF)	Male	10.9	Yes	23.9	Yes	7.21	6.0	40	3850	0.60	73
Type 1 diabetes (ICA: 80 JDF)	Female	11.1	Yes	33.7	Yes	7.36	15.3	34*	1990	-0.45	32

*BW: birth weight

*Induced premature delivery due to oligohydramnios

Table 1. Clinical characteristics of patients diagnosed with diabetes before 1 year of age. Standard deviation scores (SDS) for birth weights were calculated by comparing to the Child Growth Foundation LMS data (9). ICA, islet cell autoantibodies; JDF: Juvenile Diabetes Foundation units.

DISCUSSION

Approximately 10% of the patients with a clinical diagnosis of type 1 diabetes in this study did not have autoantibodies on initial testing or on follow-up. This is in keeping with previous reports showing that 5–20% of paediatric patients with newly diagnosed type 1 diabetes are autoantibody negative, with the proportion being higher in older children and adolescents and in non-Caucasian ethnic groups (10–12). Antibody-negative type 1 diabetes in children has a slower disease progression than antibody-positive diabetes, with preservation of residual β -cell function, lower insulin requirement, and better glycaemic control during the first year after diagnosis (13). Whether antibody-negative type 1 diabetes arises from a different pathogenic mechanism leading to β -cell failure is not currently known. Islet cell autoantibodies may disappear overtime after diabetes onset in a significant proportion of children with proven autoimmune diabetes (14). It has also been reported that a small proportion of cases might lose autoantibodies before diabetes onset (15). Thus, lack of pancreatic autoantibodies does not exclude autoimmune diabetes, especially if testing is performed during follow-up. Our study is a cross-sectional study and patients with a negative result were tested later after diagnosis

than patients with positive antibodies. This might explain that, in contrast to previous reports, patients with antibody-negative diabetes were younger at diagnosis than children with confirmed autoimmune diabetes in our cohort.

We hypothesized that a mutation in a gene implicated in monogenic diabetes could be responsible for some cases of antibody-negative type 1 diabetes. Following sequencing the most common subtypes of monogenic diabetes in 25 children with antibody-negative diabetes, we identified two patients with monogenic diabetes (8%). This supports that molecular testing in patients with antibody-negative diabetes increases the yield for identifying children with monogenic disease. Both patients had an *INS* mutation and were diagnosed with diabetes at 8 and 12 months of age. *INS* mutations are rare in unselected patients with permanent diabetes diagnosed between 6 and 12 months of age (16). Low birth weight has been associated with *INS* mutations (16) but it did not discriminate between early-onset type 1 diabetes and *INS* mutation carriers in our cohort. Absence of ketosis cannot be used as a clinical marker either, as ketosis has been previously reported in patients with an *INS* mutation (6, 8, 16-18). Therefore, lack of pancreatic antibodies might be the best single clinical criterion for genetic testing in infants diagnosed between 6 and 12 months (6, 7).

In addition, while the proband with the G32S mutation in *INS* has had similar glycaemic control over time to the four antibody-positive patients diagnosed at a similar age, the child with the R89C mutation has maintained unusually good glycaemic control (HbA1c 6-7%) with a slow increase in insulin requirements (0.2-0.4 U/kg/day). This pattern is unusual in infancy-onset type 1 diabetes and probably indicates slowly progressive β -cell destruction (Figure 1).

We did not find patients with undiagnosed *HNF1A* or *HNF4A* MODY. Making a molecular diagnosis is important, as in both forms, oral sulphonylureas are the treatment of choice. These patients have previously been shown to be misdiagnosed as type 1 diabetes and a number of different strategies have been used to identify them, including a non high-risk HLA haplotype, positive family history of diabetes and/or persistently

measurable C peptide (2-5). Our cohort originated from a paediatric cross-sectional study and therefore older children and adolescents are under-represented as those diagnosed later in childhood are quickly transferred to adult clinics. *HNF1A* and *HNF4A* mutation carriers who develop diabetes within the paediatric age range are usually diagnosed around adolescence (median age: 14 years, IQR: 12-16 years, n=215 probands diagnosed <19 years) (A.T. Hattersley and S. Ellard, unpubl. observ.). The age at diabetes onset was significantly younger in our patients, with only two having been diagnosed after 11 years. This could explain why we did not find any *HNF1A* or *HNF4A* mutations in our cohort.

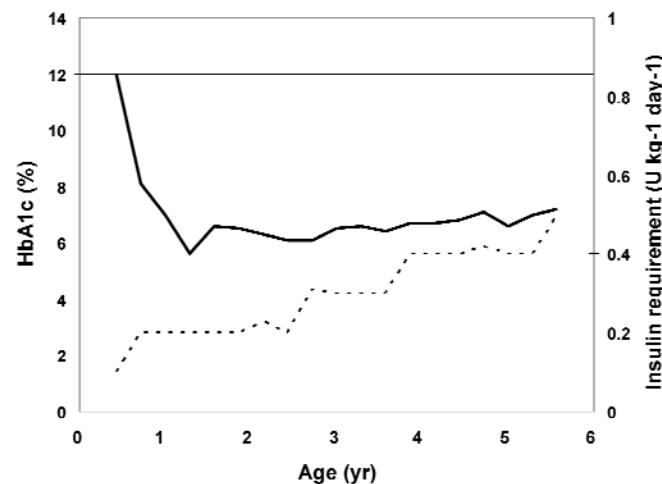


Figure 1. Glycated Haemoglobin (HbA1c) (black line) and insulin requirements (dotted line) of the patient with the R89C mutation in the *INS* gene. Apart from the initial metabolic decompensation, the patient has maintained HbA1c <7% for several years, requiring only partial replacement insulin doses. This is in keeping with the presence of an unusually long partial remission phase and is probably because of rather slow progressive destruction of insulin-producing pancreatic β -cells.

In conclusion, monogenic diabetes is uncommon in children with type 1 diabetes. However the lack of pancreatic autoantibodies can be used as an indicator for genetic testing. At present, most clinics do not routinely screen children with newly developed diabetes for autoantibodies. Further prospective studies are needed to show whether

autoantibody screening in children and adolescents with diabetes is cost-effective and should inform the need for referral for genetic testing.

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CHAPTER 3

Clinical heterogeneity in patients with *FOXP3* mutations presenting with permanent neonatal diabetes

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ABSTRACT

Objective: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome is caused by *FOXP3* mutations. We aimed to determine the prevalence, genetics and clinical phenotype of *FOXP3* mutations in a large cohort with permanent neonatal diabetes (PNDM).

Research Design and Methods: The 11 coding exons and the polyadenylation region of *FOXP3* were sequenced in 26 male subjects with diabetes diagnosed before 6 months of age in whom common genetic causes of PNDM had been excluded. Ten subjects had at least one additional immune-related disorder, and the remaining 16 had isolated diabetes.

Results: We identified four hemizygous *FOXP3* mutations in 6 of 10 patients with associated immune related disorders and 0 of 16 of those with isolated diabetes ($p=0.002$). Three patients with two novel mutations (R337Q and P339A) and the previously reported L76QfsX53 developed classical IPEX syndrome and died within the first 13 months. The novel mutation V408M was found in three cases from two unrelated families and had a mild phenotype with hypothyroidism and autoimmune enteropathy ($n=2$) or nephrotic syndrome ($n=1$) and survival to 12–15 years.

Conclusions: *FOXP3* mutations result in ~4% of cases of males with permanent diabetes diagnosed before 6 months. Patients not only have classic IPEX syndrome but, unexpectedly, may have a more benign phenotype. *FOXP3* sequencing should be performed in any male patient diagnosed with diabetes in the first 6 months who develops other possible autoimmune-associated conditions, even in the absence of full IPEX syndrome.

INTRODUCTION

Type 1 diabetes is the leading cause of diabetes among children except for those in whom diabetes is diagnosed before the age of 6 months. HLA studies have shown that patients diagnosed with diabetes within the first six months of life (permanent neonatal diabetes mellitus, [PNDM]) do not harbor high-risk HLA haplotypes and hence are very unlikely to have classic type 1 diabetes (1,2). These patients should be tested for monogenic causes of neonatal diabetes.

Mutations in *FOXP3* have been associated with a severe, early-onset, male-limited autoimmunity syndrome known as IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked; OMIM [Online Mendelian Inheritance in Man] 304930) (3-5). The gene maps to chromosome Xp11.23 and encodes a 431-amino acid protein, also named scurfin, required for the generation and functioning of CD4⁺CD25⁺ regulatory T lymphocytes. FOXP3-expressing CD4⁺ T cells are potent suppressors of self-reactive T-cell activation and proliferation, presumably via direct cell-cell interaction. Thus, lack of these cells results in an uncontrolled autoimmune reactivity in male patients with hemizygous *FOXP3* mutations (6). In keeping with an X-linked recessive mode of inheritance, heterozygous carrier females remain completely asymptomatic, but each son has a 50% risk of being affected with IPEX syndrome. One patient with IPEX syndrome due to recessive inheritance of *CD25* mutations has recently been reported (7).

Most IPEX patients described to date have developed symptoms shortly after birth or during the first 3–4 months of life. The most common findings have been enteropathy (nearly 100% of patients), diabetes (~70%), skin disease (~65%), failure to thrive (~50%), thyroiditis (~30%), and recurrent infections (~20%). Less common additional features include autoimmune cytopenias, pneumonitis, nephritis, hepatitis, vasculitis, arthritis, myositis, and alopecia as well as lymphadenopathy and splenomegaly. These disorders often appear sequentially rather than simultaneously, and

the affected organ spectrum varies substantially from patient to patient (8). The life expectancy of patients with IPEX syndrome rarely extends beyond infancy.

However, a milder phenotype has been reported in a number of patients who can live longer, sometimes into adulthood. Enteropathy was present in virtually all of them, although diabetes was frequently absent (9-11).

To our knowledge, *FOXP3* has never been systematically studied before in patients with early-onset diabetes. Hence, we aimed to explore the prevalence of *FOXP3* mutations in the largest worldwide cohort of PNDM.

RESEARCH DESIGN AND METHODS

This study was conducted in accordance with the Declaration of Helsinki, as revised in 2000. Informed consent was obtained from all patients, with parental consent given on behalf of children.

The study population originated from the International Society of Pediatric and Adolescent Diabetes (ISPAD) Rare Diabetes Study. In total, 296 patients (154 boys) with diabetes diagnosed in the first 6 months of life who were receiving insulin treatment at the time of referral were studied. A genetic cause for the disease had previously been identified in 171 subjects: *KCNJ11* in 85 patients, *INS* in 37, *ABCC8* in 28, *GCK* in 8, *EIF2AK3* in 8, *PTF1A* in 2 and *IPF1* in 1 patient (12-14; S.E. and A.T.H., unpublished data). The most common causes of PNDM had been excluded by direct sequencing in the remaining 125 patients, 70 of whom were male. Specifically, mutations in *KCNJ11* had been excluded in 68 patients, *ABCC8* mutations had been excluded in 63 patients, *INS* mutations had been excluded in 63 patients, and *GCK* mutations had been excluded in 31 patients.

Among the 70 male patients without a molecular diagnosis, we sequenced *FOXP3* in 10 subjects from 9 unrelated families on the basis of the presence of PNDM and any other immune-mediated disease. As a comparison, we also sequenced *FOXP3* in 16 further male patients with PNDM but without any associated immune disease.

DNA samples were collected in Exeter. Clinical information was obtained from hospital records with assistance from the referring clinicians. Pancreatic autoantibodies were measured locally according to the standard clinical practice. Sequence variants were tested for their presence in family members whenever a DNA sample was available.

Molecular genetic analysis

Genomic DNA was extracted from peripheral leukocytes using standard procedures. The 11 coding exons, minimal promoter, and the 3'-untranslated region of the *FOXP3* gene including the polyadenylation signal were amplified by PCR; primers and conditions are available upon request. Sequence-specific primers for each amplicon were tagged with 5' M13 tails to allow sequencing to be performed with a "universal" M13 primer.

Single strand sequencing was carried out using standard methods on an ABI 3730 sequencer (Applied Biosystems, Warrington, UK). Sequences were compared to the published sequence (NM_014009.3) using Mutation Surveyor (version 3.10). Any changes in the sequence were checked against published polymorphisms and mutations and for conservation across species.

We used a panel of microsatellites for chromosome 20q to confirm family relationships in those families where the mutation seemed to have arisen *de novo*. Patients with PNDM and other autoimmune disease in whom no *FOXP3* mutation was identified were also investigated for the presence of *CD25* mutations by sequencing of the coding region.

Molecular models

Predicted three-dimensional structures for the forkhead DNA-binding domain (DBD) (residues 335-423) of normal and variant FOXP3 proteins were generated using SWISS-MODEL (<http://swissmodel.expasy.org/>), an Internet-based tool for automated comparative protein modeling (15). Structures were visualized and images generated using either MDL Chime (Symyx Technologies) or DeepView (Swiss Pdb-Viewer) programs (16).

RESULTS

Molecular genetics

We identified four different mutations in *FOXP3* in six male subjects with PNDM from five unrelated families, all of whom presented with an associated immune-mediated disease (see below). None of the 16 patients with isolated PNDM had a variant in the *FOXP3* sequence (Fisher's exact test; $p=0.002$). No *CD25* mutations were identified in probands lacking *FOXP3* mutations.

There were 3 novel *FOXP3* missense mutations: R337Q (c.1010G>A; p.Arg337Gln), P339A (c.1015C>G; p.Pro339Ala) and V408M (c.1222G>A; p.Val408Met), the latter being present in 3 patients (including monozygotic twins) from two unrelated pedigrees (Figure 1). The affected amino acid residues were conserved among species as well as in several other members of the forkhead transcription factor superfamily (data not shown). None of them was found in 510 European Caucasian or in 147 Turkish control X chromosomes. Only one of the mutations had previously been reported (c.227delT; p.Leu76GlnfsX53). It introduces a frameshift and generates a premature stop codon (17, 18). The inheritance within families of the mutations is in keeping with an X-linked inheritance as shown in Figure 1.

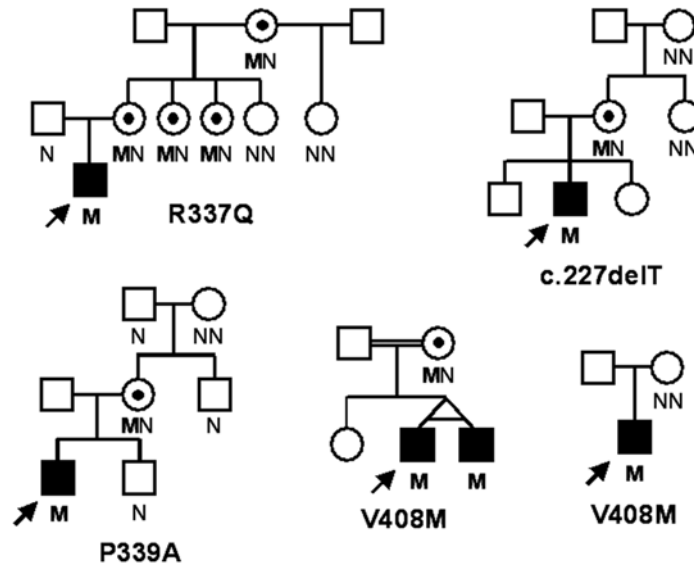


Figure 1. Pedigrees for families with individuals harboring a *FOXP3* mutation. Filled symbols represent patients with early-onset diabetes; black dots represent asymptomatic female carriers. The genotype is shown underneath each symbol; M and N denote mutant and wild-type alleles, respectively. An arrow indicates the proband.

Clinical features

The clinical information from patients bearing a *FOXP3* mutation is shown in Table 1. There were no significant clinical differences (including age at diabetes onset, birth weight, or type of associated autoimmune disease) between those who were positive and negative for *FOXP3* mutations (data not shown). Classic IPEX syndrome was present in three of the six patients, all of whom died by 13 months. In contrast, all three patients with the V408M mutation had a milder phenotype as well as prolonged survival (still alive at age 12–15 years).

Diabetes was the presenting feature in all subjects. Age at diagnosis varied from the first day of life to 3.5 months and did not correlate with the outcome of the disease. Birth weight was a feature of disease severity as it was markedly reduced only in the two patients who died early in infancy. Antibodies against β -cell antigens were reported to be unequivocally present just in one patient; in this patient results were negative at the time diabetes was diagnosed and became positive only during follow-up.

Subject	Country	Gestational age (wk)	Birth weight (g)	Age at onset of diabetes	Glucose at diagnosis (mmol/l)	Ketosis at diagnosis	<i>FOXP3</i> mutation	Current status	Associated clinical features
I	Czech Republic	36	2,400 (-0.74 SDS)	2 days	15	Negative	V408M	Alive at 15 years	Mildly elevated TSH at 3 years (thyroid antibodies negative); nephrotic syndrome at 6 years (good response to steroids); transient ischemic attack at 13 years (normal brain MRI scan); GAD and IA2 antibodies negative at 14 years; No diarrhea or malabsorption (anti-endomysial and anti-transglutaminase IgA negative); current A1C 10%; chronic diabetes complications (microalbuminuria and polyneuropathy)
IIa	Germany (Turkish)	40	3,720 (-0.34 SDS)	3 weeks	48.5	NA	V408M	Alive at 12 years	Autoimmune hypothyroidism at 1 year (TSH: 193 U/ml; TPO Abs 3,000 units/l; TG Abs 2,000 units/l); enteropathy at 3 years (anti-enterocyte, anti-gliadin IgG, anti-endomysium and anti-transglutaminase IgA, parietal cell antibodies all positive; villous atrophy on jejunal biopsy); recurrent respiratory and gastrointestinal infections; mucocutaneous candidiasis; ICA and GAD Abs negative; current A1C 8.6%; no chronic diabetes complications; mild intellectual impairment (IQ 74)
IIb	Germany (Turkish)	40	3,750 (-0.40 SDS)	3.5 months	29.7	NA	V408M	Alive at 12 years	Autoimmune hypothyroidism at 1 year (TSH: 177 U/ml; TPO Abs: 3,000 units/l; TG Abs 2,000 units/l); enteropathy since age 3 years (anti-enterocyte, anti-gliadin IgG, anti-intrinsic factor and parietal cell Abs positive, anti-endomysial and antitransglutaminase IgA Abs negative; no villous atrophy on jejunal biopsy); recurrent respiratory and gastrointestinal infections; mucocutaneous candidiasis; hypochromic microcytic anemia since age 10 years; ICA and GAD Abs negative; current A1C 8.5%; no chronic diabetes complications; mild intellectual impairment (IQ 80)
III	Argentina	38 + 3	3,180 (-0.12 SDS)	30 days	40	DKA	R337Q	Died at 13 months	ICA and GAD Abs negative at diagnosis, positive at 4 months (120 JDF units and 38 units/l, respectively); watery diarrhea at 3 months (severe villous atrophy, absent antienterocyte antibodies); serum IgE 2,266 units/ml at 3 months; normal thyroid function
IV	Germany	39 + 3	1,590 (-3.67 SDS)	1 week	25.6	NA	P339A	Died at 5.5 months	Maldigestion; cholestasis (attributed to parenteral nutrition); eczema; euthyroid thyroiditis at 6 weeks (TPO Abs 117 units/l; TG Abs 253 units/l)
V	England	33 + 5	1,250 (-2.65 SDS)	1 day	23	NA	L76QfsX53	Died at 8 months	Absent GAD, ICA, and IA2 antibodies; watery diarrhea; low fecal elastase at 2.5 months; initially normal (small portion of pancreatic tissue in abdominal MRI scan); anemia; neutropenia; thrombocytopenia; increased IgE (132 units/ml); thyroid dysfunction (negative thyroid antibodies); recurrent respiratory tract infections and sepsis

Ab, antibody; DKA, diabetic ketoacidosis; IA2, insulinoma-associated protein 2; ICA, islet cell antibody; JDF, Juvenile Diabetes Foundation; NA, not available; SDS, standard deviation score; TG, thyroglobulin; TPO, thyroperoxidase; TSH, thyroid-stimulating hormone.

Table 1. Clinical characteristics of the patients with a *FOXP3* mutation.

Gastrointestinal symptoms developed in five of six patients, although both the timing and the clinical severity varied widely. Interestingly, one of the most severely affected infants (Table 1, proband V) had diarrhea with a low fecal elastase and a magnetic resonance imaging (MRI) scan performed at 4 months showed only a tiny remnant of pancreatic tissue (Figure 2). Thyroid disease was also found in five of six patients, but again it ranged between very mildly elevated thyroid-stimulating hormone levels without any detectable thyroid antibodies to severe autoimmune hypothyroidism. Proband I (hemizygous for V408M) did not suffer from any gastrointestinal problem and only had a steroid-sensitive nephrotic syndrome that was considered to represent minimal change disease by the attending nephrologist.

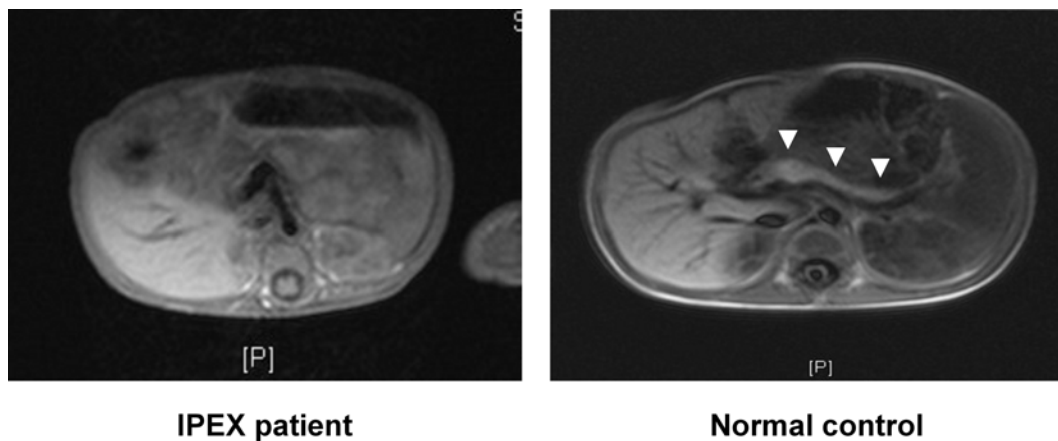


Figure 2. Abdominal MRI scan showing the absence of recognizable pancreatic tissue in one of the patients with classic IPEX syndrome (c.227delT, p.Leu74GlnfsX53 mutation). White arrowheads point to the pancreas in a control subject.

Molecular models

Inspection of the predicted structures of the DBD of FOXP3 suggested that all three novel missense *FOXP3* mutations are likely to have deleterious effects on protein function and therefore be pathogenic, although to varying degrees. In normal FOXP3, the side chain of arginine 337 is predicted to make close contact with the DNA backbone (Figure 3A, top panel). The presence of a strong basic residue at this position is

conserved in all forkhead transcription factors, and it contributes to DNA binding through both hydrogen bonding and electrostatic interactions. The R337Q substitution destroys this close contact with the DNA backbone (Figure 3A, bottom panel) and is predicted to cause a significant loss of positive charge at the DNA-binding surface of FOXP3 (Figure 3B). Taken together, the data strongly suggest that the R337Q mutation will result in a significant loss of DNA binding affinity.

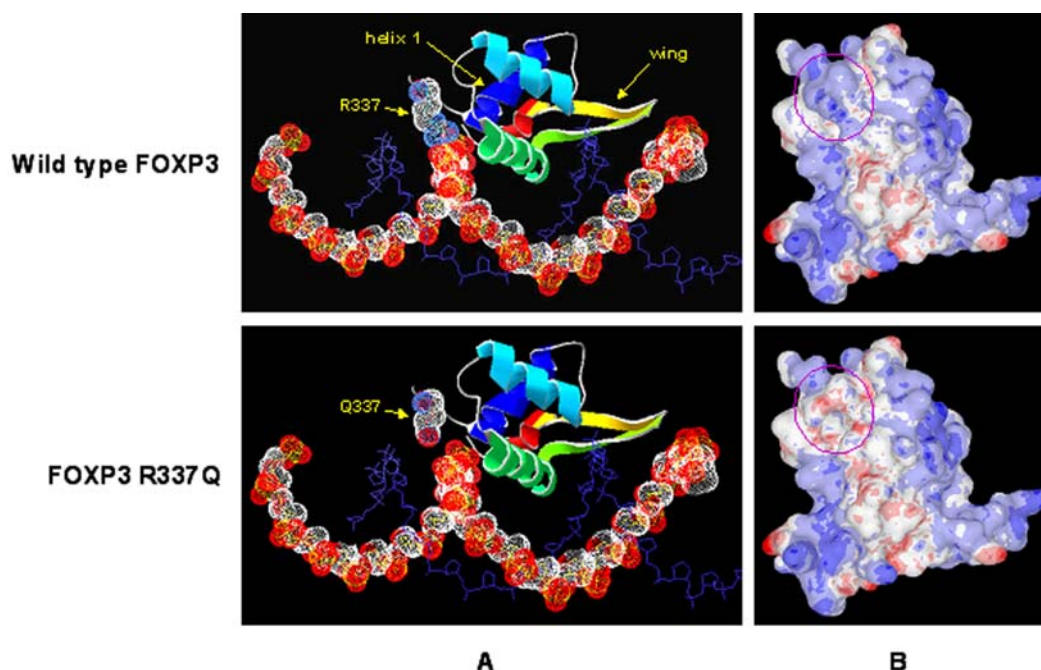


Figure 3. Predicted structures of the DBD of normal and R337Q FOXP3. A: Predicted interaction of FOXP3 with DNA. FOXP3 is represented in ribbon form, colored blue (NH₂-terminal) to red (COOH-terminal), except for residue 337 (labeled) that shows the van der Waals radii of backbone and side chain atoms as dotted surfaces (white, carbon; blue, nitrogen; red, oxygen). Other features referred to in the text (helix 1 and the wing region) are labeled; the main DNA recognition helix (helix 3, green) lies in the major groove of the DNA. The DNA strand predicted to contact FOXP3 R337 is represented as a space-filling model, showing the van der Waals radii of the backbone atoms (white, carbon; red, oxygen; yellow, phosphorus). For clarity, each of the second DNA strands is shown only as a stick model of the backbone (blue). B: Molecular surface of FOXP3, showing areas of positive (blue) or negative (red) electrostatic potential. The structure shown in A has been rotated upward to look toward the DNA-binding surface; the position of residue 337 is indicated by the purple oval, and the DNA strands have been omitted for clarity. Structures were visualised and molecular surfaces calculated using DeepView (A) or MDL Chime (B) programs.

Proline 339 lies in a gamma-turn between Arg³³⁷ and helix 1 of the DBD. Substitution of this residue by alanine (as in the P339A mutation) is likely to affect the topology of Arg³³⁷ and/or helix 1 relative to the DNA and so would also be expected to have a deleterious effect on DNA binding affinity.

Valine 408 lies in a β -turn at the COOH-terminal of wing 1; in the predicted structure of FOXP3 its side chain points away from the DNA-binding surface and towards the side chain of arginine 347 in helix 1 (data not shown), although there is no apparent contact between the two side chains. Interestingly, replacing Val⁴⁰⁸ with the longer methionine is predicted to allow van der Waals contact between the two side chains (data not shown). This increased interaction between wing 1 and helix 1 is likely to have some effect on protein flexibility within the DBD, and it is reasonable to suspect that reduced flexibility in the DBD of FOXP3 will have an impact on DNA binding affinity. However, given the weak nature of van der Waals interactions, such effects are likely to be relatively modest, and the V408M substitution has no apparent effect either on the predicted hydrogen bond between the nearby residue Tyr⁴⁰⁶ and the DNA backbone or on surface charge distribution (data not shown). Therefore, the effects of the V408M substitution on protein structure are milder than the other mutations which is consistent with the mild phenotype observed among subjects with this mutation.

CONCLUSIONS

Our systematic search for *FOXP3* mutations in a large cohort of male probands with PNDM in whom the common causes had been excluded has shown that mutations are common in males who subsequently develop possible immune-mediated disorders (6 of 10 [60%]) but are absent or very rare in those who have isolated diabetes (0 of 16 [0%]). We show that there is clinical heterogeneity with three of the six mutation carriers having a severe phenotype and dying by 13 months, whereas the other three are still alive at 12–15 years.

We report on four different mutations, including three novel mutations. The previously reported single base pair deletion at position 227 generates a frameshift and introduces a premature stop codon. If the mutant mRNA evades nonsense mediated decay, the resulting protein would lack the forkhead as well as the zinc finger and the leucine zipper domains and is predicted to be completely inactive. Consistent with this null mutation, the patient presented with classic IPEX syndrome and died within the first year of life. The likely pathogenicity of the three novel missense mutations was explored by using molecular modeling software. All of them are located within the forkhead domain of FOXP3; their predicted structural effects are likely to alter function of the protein and can be broadly correlated with the observed phenotype. The pathogenicity of R337Q is further supported as a different substitution at position 337 (R337P) has previously been described in a patient with IPEX syndrome (19). A substitution of asparagine for aspartic acid at position 409 of FOXP3 (adjacent to the site of the V408M mutation) also produces IPEX syndrome (20). Thus, we believe that the three novel mutations are likely to be responsible for the observed phenotypes.

Permanent diabetes was the presenting feature in the six patients with a *FOXP3* mutation, although age at diagnosis varied. There was no correlation between the age at presentation and the clinical outcome of the patients; however, there was a suggestion that low birth weight, a reflection of intrauterine insulin secretion, was a prognostic marker: two of the three patients who died within the first thirteen months of life as a result of a severe IPEX syndrome were born with severe intrauterine growth retardation. Low birth weight might be a marker of poor prognosis among patients with *FOXP3* mutations.

Autoantibodies against β -cell antigens were only persistently found in one of our six affected children, and in this child they were not present at diagnosis, suggesting that the main pathogenic mechanism leading to β -cell destruction is mediated by activated T lymphocytes. A systematic investigation of all possible beta-cell autoantibodies was not performed in these children, and serum was not available for a retrospective analysis in a central laboratory. Therefore the complete lack of antibodies cannot definitely be

confirmed. However, our results show that antibodies against β -cell proteins as they are used in routine clinical practice may be absent in patients with proven *FOXP3* mutations. Thus, the absence of pancreatic autoantibodies should not be taken as a screening test to exclude the possibility of a *FOXP3* mutation.

One patient with a classic IPEX syndrome only had a small remnant of pancreatic tissue seen on an abdominal MRI scan and had evidence of evolving exocrine pancreatic dysfunction, suggesting the development of pancreatic atrophy. Although both mild pancreatic exocrine insufficiency and a reduced pancreas volume are relatively common findings in long-standing classic type 1 diabetes they are not as severe as in this patient. It is possible that a massive autoimmune response against the pancreas might be responsible for both PNDM and pancreatic atrophy in this patient as seen in maternal enteroviral infection (21).

There was considerable clinical heterogeneity among the patients with *FOXP3* mutations. Three patients with classic IPEX syndrome died during the first 13 months of life despite the use of immunosuppressive therapy. However, the phenotypic spectrum is extended from that previously described in the three patients with the V408M mutation. Permanent neonatal diabetes was the only clinical feature in two twin brothers from one of the pedigrees with this mutation until the age of 1 year, when autoimmune hypothyroidism was diagnosed in both of them. Subsequently, they both developed recurrent diarrhea, and autoimmune enteropathy was diagnosed on the basis of positive antienterocyte antibodies in both twins. They had multiple antibodies indicating gastrointestinal autoimmunity (Table 1). Twin IIa had a jejunal intestinal biopsy showing villous atrophy, but the second twin (IIb) had a normal jejunal biopsy. Interestingly, they both developed very well on a gluten-free diet. These patients are now 12 years old, and no other significant problems have been detected except that both have intellectual impairment (IQ of 74 and 80, respectively). The other patient with a V408M mutation has the mildest phenotype associated with a *FOXP3* mutation to date. Although diabetes was diagnosed 2 days after birth, no other evidence of immune dysfunction has been detected apart from an episode of nephrotic proteinuria at 6 years. Clinical remission was achieved

on steroid treatment, and the disease has not relapsed. The patient is currently 15 years, and no other autoimmune problems have arisen since then. Nephrotic syndrome and classic type 1 diabetes can be associated, indicating a shared immunological basis for both disorders (22, 23), and nephrotic syndrome has previously been described in a patient with classic IPEX syndrome (24). We therefore believe there may be a causal link between both diabetes and nephrotic syndrome and the presence of the V408M mutation in our patient.

IPEX syndrome is thought to be an extremely rare condition with only approximately 50 cases reported in the literature. As with any other X-linked recessive disorder with reduced survival, IPEX frequently presents as a single case either caused by *de novo* or maternally inherited mutations. Patients with a mild presentation are likely to have been under-reported, so the true incidence of the disease might actually be higher than is currently perceived. A clinical diagnosis of IPEX syndrome was made in only one of our patients prior to the genetic analysis, whereas the others were referred for genetic testing because of their PNDM. The varied clinical spectrum resulting from mutations in *FOXP3* emphasizes the need of be aware of this possibility in male patients with PNDM who develop any other immune-mediated disorder later in life.

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CHAPTER 4

Permanent neonatal diabetes caused by a homozygous nonsense mutation in the glucokinase gene

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ABSTRACT

Glucokinase deficiency is an unfrequent cause of permanent neonatal diabetes (PND), as only seven patients have been reported, either homozygous for a missense or frameshift mutation or compound heterozygous for both of them. We report here the first known case caused by a homozygous nonsense mutation (Y61X) in the glucokinase gene (*GCK*) that introduces a premature stop codon, generating a truncated protein that is predicted to be completely inactive as it lacks both the glucose- and the adenosine triphosphate-binding sites. The proband, born to consanguineous parents, was a full-term, intrauterine growth-retarded male newborn who presented with a glycaemia of 129 mg/dL (7.16 mmol/L) on his second day of life, increasing thereafter up to 288 mg/dL (15.98 mmol/L) and 530 mg/dL (29.41 mmol/L) over the next 24 h, in the face of low serum insulin (<3 mIU/mL; <20.83 pmol/L). He was put on insulin on the third day of life. Insulin has never been discontinued since then. The patient was tested negative for antiinsulin and islet cell antibodies at age 5 months. His father had nonprogressive, impaired fasting glucose for several years. The mother was found to be mildly hyperglycaemic only when her glucose was checked after the child was diagnosed. In conclusion, biallelic *GCK* loss should be considered as a potential cause of PND in children born to consanguineous parents, even if they are not known to be diabetic at the time of PND presentation.

INTRODUCTION

There is increasing evidence indicating that most cases of insulin-requiring hyperglycaemia diagnosed in infants less than 6 months old must be considered as the consequence of underlying monogenic disorders until proven otherwise (1–4). The incidence of this condition, for which the term ‘monogenic diabetes of the infant’ has been proposed, seems to be higher than previously thought affecting approximately 1 in 100,000 live births (4).

The outcome of this condition is varied as some infants require insulin treatment for life (permanent neonatal diabetes, PND), whereas others present a remission (transient neonatal diabetes, TND) that may be either transient with subsequent recurrence around puberty or apparently permanent (5). TND is associated with imprinting abnormalities in chromosome 6 and, to a lesser extent, with mild activating mutations in either of the two genes encoding the adenosine triphosphate (ATP)-sensitive potassium channel of the beta cell, namely *ABCC8* (encoding the SUR1 subunit) or *KCNJ11* (encoding the Kir6.2 subunit) (2, 3). In contrast, PND is mainly produced by severe activating *KCNJ11* mutations, although insulin gene mutations have been also recently reported as an important cause of PND (6), and *ABCC8* mutations have been involved in a few cases (7). In addition, a number of other less frequent causes have been also described (8).

It is well known that the glucose-sensing enzyme glucokinase (GCK, hexokinase 4; E.C.2.7.1.2), encoded by glucokinase gene (*GCK*) (7p15.3–p15.1), plays a central role in glucose homeostasis. Activating heterozygous *GCK* mutations have been identified in several patients with persistent hyperinsulinemic hypoglycaemia of infancy, whereas inactivating heterozygous *GCK* mutations cause the mildest form of maturity-onset diabetes of the young, type 2 (MODY2) (9). In contrast, homozygous or compound heterozygous inactivating *GCK* mutations have been described in a few cases of PND (10–12).

We report a new case of PND caused by a complete GCK deficiency secondary to a homozygous *GCK* mutation and compare the patient's clinical phenotype with those previously published.

PATIENT REPORT

A male newborn was admitted to the neonatal intensive care unit (NICU) because of respiratory distress and intrauterine growth restriction (IUGR). He was born at 41-wk gestation to an 18-yr-old primigravida woman by spontaneous vaginal delivery after an uncomplicated pregnancy. Thick meconium was noted during labour. Nevertheless, his Apgar scores were 8 and 9 at 1 and 5 min, respectively, and no resuscitation was necessary. Birth weight (BW) was 1800 g [-3.80 standard deviation scores (SDS)], length 46 cm (-2.17 SDS) and head circumference 33 cm (-1.92 SDS). The infant developed mild respiratory distress, otherwise the physical examination shortly after birth was completely normal.

On admission to the NICU, serum glucose concentration was low (37 mg/dL; 2.05 mmol/L), and the infant was given 10% dextrose at a rate of 80 mL/kg/d (10 mg/kg/min). Respiratory distress improved spontaneously within the next few hours, and transient tachypnoea was considered to be the most likely diagnosis. By the second day of life, the glycaemia was 129 mg/dL (7.16 mmol/L). Subsequent glucose concentrations over the next 24 h increased to 288 mg/dL (15.98 mmol/L) and 530 mg/dL (29.41 mmol/L), and glycosuria without ketonuria was noted. Serum insulin concentration was low (<3 mU/mL). At this time, the dextrose infusion rate was 12.5 mg/kg/min. The intravenous (IV) solution was changed to 5% dextrose, but the glycaemia remained high, and IV insulin was started on the third day of life (initial rate: 0.1 U/kg/h). By that time, the infant had passed several hypocholic stools and was noted to be mildly jaundiced. Serum conjugated bilirubin concentration was elevated (4.5 mg/dL, accounting for 85% of total serum bilirubin), and liver function tests were consistent with a cholestatic syndrome

(aspartate aminotransferase: 43 U/L, alanine aminotransferase: 44 U/L and g-glutamyl transpeptidase: 216 U/L). He underwent several specific investigations (urine cultures, abdominal and brain ultrasound, ophthalmologic examination, skeletal X-ray survey, serologic tests for perinatal infections, metabolic and endocrine screening, serum α 1-antitrypsin and immunoreactive trypsinogen level, and serum total cholesterol), but none of them was informative, and the usual causes of neonatal cholestasis were ruled out. He was fed with an elementary formula and received treatment with phenobarbital for 7 d, after which cholestasis resolved. By age 23 d, the patient developed high temperature, poor feeding and poor reactivity and hyperglycaemia worsened. He received broad-spectrum antibiotic treatment for 10 d as blood cultures turned positive for *Escherichia coli* infection.

At the age of 1 month, the patient was transferred to subcutaneous insulin. Since then, the treatment was never discontinued. Fasting C-peptide level was 0.1 ng/mL at 3 months of age (normal range: 0.3–5.4 ng/mL). By that time, the infant had experienced a significant catch-up growth [weight: 5,000 g (-1.87 SDS), length 57 cm (-1.62 SDS) and head circumference 39.5 cm (-1.05 SDS)]. Anti-insulin and islet cell antibodies were negative at age 5 months. An abdominal computerized tomography (CT) scanning performed at 8 months revealed a normal pancreas, thus excluding pancreatic agenesis as the cause of his PND. Basal (0.1 ng/mL, 1 month; 0.9 ng/mL, 9 months) and glucagon-stimulated (0 min, 0.3 ng/mL; 6 min, 0.1 ng/mL; 22 months) C-peptide levels determined during the first 2 yr of life confirmed very low basal and stimulated endogenous insulin secretion. Because of poor family support and psychosocial difficulties, the infant was managed on an inpatient basis until he was 11.5 month old, when he was discharged on a twice-daily insulin regimen (0.8 U/kg/d). His weight was 8.815 g (-0.98 SDS), his length 72.5 cm (-0.55 SDS) and his head circumference 45 cm (-1.65 SDS). Psychomotor development was considered completely normal. During admission, he suffered a number of infections as follows: otitis, bronchiolitis and pneumonia at 5 months; urinary tract infection at 6 months; parotitis at 8 months and gastroenteritis at 11 months. A voiding cystourethrogram was normal, and vesicoureteral reflux was excluded. His

postnatal growth and pubertal development and progress were normal: Tanner II at age 12.2 yr; height 147 cm (-0.08 SDS), BW 37.6 kg (-0.26 SD); Tanner IV at age 16.8 yr; height 167 cm (-1.09 SDS) and BW 62 kg (-0.33 SDS). The patient is currently 17 yr old. Excluding diabetes, he is otherwise a healthy adolescent with no chronic complications related to diabetes. Optimal metabolic control has never been achieved, mainly because of poor compliance with diet and insulin treatment, but it improved after glargine introduction when the child was 13 yr old (mean HbA1c \pm SDS: $9.6 \pm 1.3\%$ vs. $7.9 \pm 0.5\%$ before and after glargine, respectively). He has presented to the emergency room on several occasions with moderate to severe hypoglycaemia. Neither hepatic dysfunction nor frequent infections have been present since the first hospital discharge. Current insulin dose is 1.26 U/kg/d (40% as glargine at bedtime and 60% as aspart analogue before meals). No alternative or simultaneous treatment with sulfonylureas has been attempted.

The family tree reveals parental consanguinity (paternal and maternal great grandmothers were siblings; Figure 1). Several relatives on both sides have mild fasting hyperglycaemia that does not need any treatment (Figure 1), resembling a MODY2 phenotype.

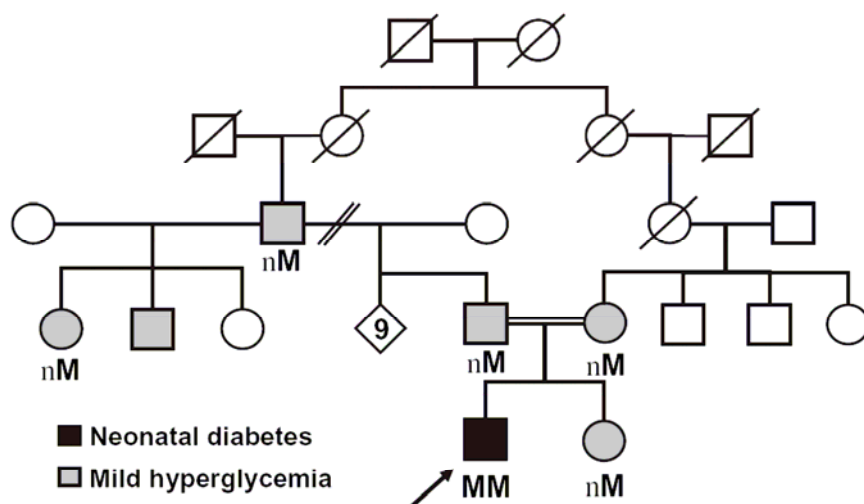


Figure 1. Family pedigree showing co-segregation of hyperglycaemia and Y61X mutation in GCK. Mutation status is denoted by M for mutant allele and n for normal allele.

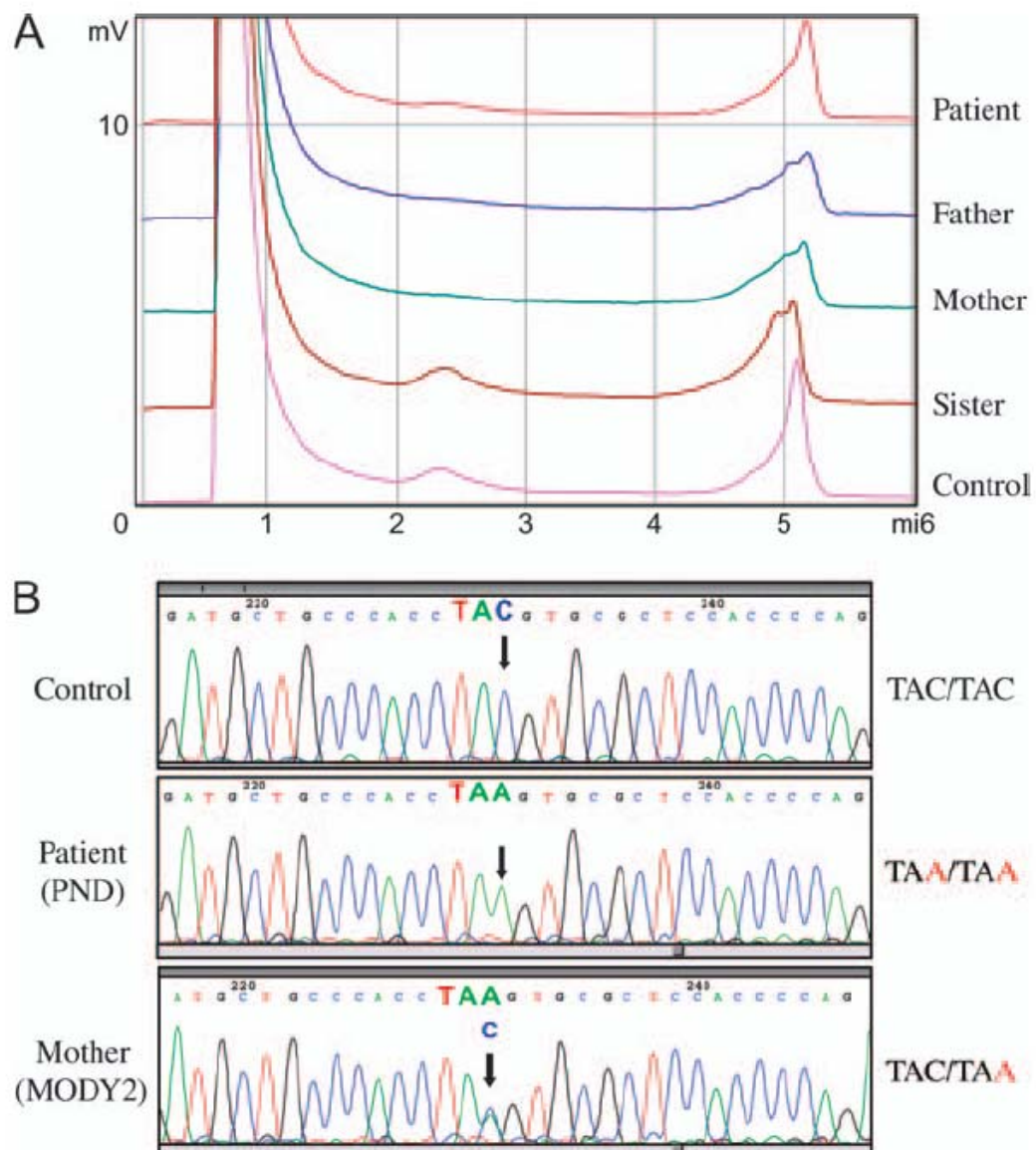


Figure 2. (A) Denaturing high performance liquid chromatography analysis of the *GCK* exon 2 including amplicon of all family members and a control showing the specific chromatographic profiles for the wild-type allele (control) and the homozygous and heterozygous carriers of the c.183C>A mutation at 62.8°C. (B) Direct sequencing of *GCK* exon 2 showing the homozygous and heterozygous nonsense mutation TAC>TAA resulting in the premature stop codon Y61X.

The coexistence in this patient of PND, parental consanguinity and a family history of mild hyperglycaemia prompted us to look for mutations in *GCK*. The study was approved by the local Ethics Committee. After obtaining written informed consent from the affected subject(s) and/or parents, DNA was extracted from peripheral lymphocytes

from the patient and several relatives, as previously described (13). The coding sequences, intron–exon boundaries and known regulatory regions of *GCK* were amplified by PCR using specific primers and screened for mutations by denaturing high performance liquid chromatography (DHPLC) (Transgenomic Wave 3500HT, Transgenomic Inc, Omaha, NE, USA), and subsequent sequencing of any identified heteroduplex or homoduplex variant was performed using the BigDye Terminator V3.1 kit (Applied Biosystems, Foster City, CA, USA) on a ABI 3100 Genetic analyzer. Further information on primer sequences and DHPLC conditions is available upon request. The proband was found to be homozygous for a nonsense *GCK* mutation located in exon 2, c.183C>A (TAC>TAA; Y61X), which introduces a premature stop codon at position 61 (Figure 2). Both parents and several hyperglycaemic relatives from whom DNA was available were heterozygous for the mutation, thus showing that the mutation co-segregated with mild hyperglycaemia in the heterozygous status (Figures 1 and 2).

DISCUSSION

Here, we describe a patient with PND caused by a homozygous nonsense Y61X mutation in *GCK*. The mutation introduces a premature stop codon at position 61, generating a truncated protein that is predicted to be completely inactive as it lacks both the glucose- and the ATP-binding sites. The mutation affects both the hepatic and the neuroendocrine isoforms of the enzyme for which the patient must be considered as having a complete *GCK* deficiency. The same mutation has been recently reported in a Spanish MODY2 family in the heterozygous state. The mutation co-segregated with the phenotype and was not present in 200 control chromosomes (14).

Since its first description in 2001 (10), complete *GCK* deficiency has been previously described in only seven patients (Table 1). Although, early-onset neonatal diabetes and IUGR were present in all of them, our patient showed some unique and interesting features. First, hypoglycaemia developed shortly after birth. This could be

explained by the fact that both insulin and hepatic GCK (lacking in our patient) are necessary for prenatal glycogen accumulation in the liver. It is well accepted that hepatic glycogenolysis is the main source of glucose for the newborn immediately after birth, before oral feeding is successfully established (15). In accordance with this, there is a significant reduction in liver glycogen stores in GCK-deficient knockout mouse (16). Moreover, meconium-stained amniotic fluid indicates some foetal distress, which is considered a further risk factor for early neonatal hypoglycaemia (17).

GCK mutations	Gender	Origin	GA (wk)	BW (g)	Ref.
M210K/M210K	Female	Norway	36	1670	6
T228M/T228M	Female	Italy	38	1650	6
A378V/A378V	Female	Turkey	33	1550	7
IVS8+2T→G/IVS8+2T→G	Male	Israel (Arabic)	40	1900	7
IVS8+2T→G/G264S	Male	Israel (Arabic)	38	1870	7
R397L/R397L	Female	UK (Pakistani)	36	1760	8
T168A/T168A	Male	Turkey	37	1400	18
Y61X/Y61X	Male	Spain	41	1800	-

Table 1. Reported patients with permanent neonatal diabetes caused by complete glucokinase deficiency. GA: gestational age; BW: birth weight.

Second, our patient developed transient unexplained cholestasis. Although PND and biliary tract malformations can coexist (18), this seems not to be the case as abdominal ultrasound and CT scans did not detect any abnormality. However, perinatal asphyxia has been associated with TND (19). Recurrent hypoglycaemia and unexplained neonatal cholestasis can be also found in patients with hypopituitarism (20). However, the patient's postnatal growth and pubertal development were normal, which excludes this possibility.

Whether GCK deficiency in tissues other than pancreas and liver has any functional relevance in these patient remains to be explored. Hypothalamic and pituitary GCK activity is especially interesting in this respect. Counter-regulatory hormonal response to hypoglycaemia is activated at a higher plasma glucose concentration in MODY2 patients (21), which could provide a relative protection against the development of severe hypoglycaemia. This has not yet been studied in detail in humans with complete GCK deficiency in whom the set point for hypoglycaemia counterregulation may be even higher. However, serum glucagons levels have been reported to be either in the upper normal or above the normal range in the face of hyperglycaemia in three of the studied patients, which indicates an excessive production of this hormone by GCK-expressing pancreatic α -cells. Although attempts to selectively abolish hypothalamic GCK activity in mice have failed, it has been shown that GCK haploinsufficiency does affect reproductive function and energy metabolism in mice (22). Our patient has presented with severe hypoglycaemia several times, and his pubertal development has been completely normal. Unfortunately, our patient's lack of interest in participating in further clinical studies impeded us to perform a more in-depth evaluation of this case.

As GCK is considered a key element of the glucose-stimulated insulin release, patients with PND caused by biallelic *GCK* loss have been thought to need insulin injections for life. Nevertheless, although no sulfonylurea treatment could be attempted in our patient, high dose glibenclamide treatment has been recently shown to stimulate endogenous basal insulin secretion and to improve metabolic control in a patient with PND caused by a homozygous *GCK* mutation, though the need for insulin persisted (23). Furthermore, Bakri et al. recently reported the case of a patient presenting with compound heterozygous *GCK* mutations, in whom one of the alleles probably retains certain enzymatic activity, who was successfully transferred to repaglinide (24). Also, the therapeutic use of GCK activators is being studied for use in patients with type 2 diabetes (25), and might offer some benefit for selected patients with GKC-related PND and complete although dysfunctional GCK proteins.

In summary, we present a new case of PND associated to a complete *GCK* deficiency caused by a homozygous nonsense mutation in *GCK*. Although biallelic *GCK* loss accounts only for a small fraction of patients with PND (26, 27), it must be considered as a potential cause, especially when both parents have mild hyperglycaemia and consanguinity is present. As *MODY2* is usually a silent disorder, testing fasting blood glucose in the parents of every infant with neonatal diabetes should be a must, even if there is not a family history of known diabetes (12).

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CHAPTER 5

Wolcott-Rallison syndrome is the most common genetic cause of permanent neonatal diabetes in consanguineous families

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ABSTRACT

Context and Objective: Mutations in *EIF2AK3* cause Wolcott-Rallison syndrome (WRS), a rare recessive disorder characterized by early-onset diabetes, skeletal abnormalities and liver dysfunction. Although early diagnosis is important for clinical management, genetic testing is generally performed after the full clinical picture develops. We aimed to identify patients with WRS before any other abnormalities apart from diabetes are present and to study the overall frequency of WRS among patients with permanent neonatal diabetes.

Research Design and Methods: The coding regions of *EIF2AK3* were sequenced in 34 probands with infancy-onset diabetes with a clinical phenotype suggestive of WRS (n=28) or homozygosity at the WRS locus (n=6).

Results: 25 probands (73.5%) were homozygous or compound heterozygous for mutations in *EIF2AK3*. Twenty of the 26 mutations identified were novel. Whilst a diagnosis of WRS was suspected before genetic testing in 22 probands, three patients with apparently isolated diabetes were diagnosed after identifying a large homozygous region encompassing *EIF2AK3*. In contrast to non-consanguineous pedigrees, mutations in *EIF2AK3* are the most common known genetic cause of diabetes among patients born to consanguineous parents (24% vs. <2%). Age at diabetes onset and birth weight might be used to prioritize genetic testing in the latter group.

Conclusions: WRS is the commonest cause of PNDM in consanguineous pedigrees. Besides testing patients with a definite clinical diagnosis, *EIF2AK3* should be tested in patients with isolated neonatal diabetes diagnosed after 3 weeks of age from known consanguineous families, isolated populations or countries where inbreeding is frequent.

INTRODUCTION

Wolcott-Rallison syndrome (WRS, OMIM #226980) is a rare autosomal recessive multi-systemic disorder due to biallelic mutations in *EIF2AK3*, the gene encoding the eukaryotic translation initiation factor 2- α kinase 3 (1). This transmembrane enzyme, also known as PERK or PEK, localizes exclusively in the endoplasmic reticulum (ER) and it is activated by the accumulation of unfolded proteins in the ER lumen during stress, resulting in phosphorylation of the α -subunit of the eukaryotic initiation factor 2 (eIF2 α) at residue Ser51 and down-regulation of protein synthesis (2). Lack of PERK activity leads to cell death by apoptosis in a number of different tissues, as clearly shown in two independent knockout mice (3, 4). The high level expression of *EIF2AK3* in both beta cells and bone tissue (4) explains the development of early-onset diabetes mellitus and skeletal abnormalities in virtually all patients with WRS, and hence their association is considered a cornerstone for diagnosing this rare clinical entity (5-7). However, the gene is expressed at lower levels in several other tissues which determines a number of other inconsistently present features (8, 9). Importantly, most patients develop chronic or intermittent acute liver dysfunction sometimes leading to hepatic failure. Genetic testing for this condition is usually delayed until the full clinical picture is evident and a clinical diagnosis of WRS made (9).

Permanent diabetes mellitus diagnosed within the first 6 months of life (permanent neonatal diabetes, PNDM) is a rare disorder likely to be monogenic rather than autoimmune (10, 11). It is estimated to affect approximately 1 in 215,000 live births (12). Most patients have heterozygous activating mutations in the *KCNJ11* and *ABCC8* genes encoding potassium ATP channel subunits Kir6.2 (13) and SUR1 (14, 15) or heterozygous mutations in the preproinsulin (*INS*) gene (16-17). In addition, a number of rare recessive causes of PNDM have been described, including *INS* (18), *ABCC8* (19), *GCK* (20), *IPF1* (21), *PTF1A* (22), *GLIS3* (23), *SLC2A2* (24), and *SLC19A2* (25).

Homozygosity mapping, performed by total genome scan with polymorphic markers in individuals whose parents are related, has been extensively used to identify the gene responsible for a recessive disorder. It assumes that a homozygous mutation in a recessive disease gene segregates twice to the affected child from a common ancestor through both the maternal and the paternal lines and is hence “identical by descent (IBD)”. The homozygous mutation will be embedded in a chromosomal segment which has not undergone recombination. Therefore SNP (single nucleotide polymorphism) markers in that segment will also be homozygous by descent (26). This short segment of homozygosity by descent can be easily detected and will harbor the disease gene. Taking into account that WRS is a recessive disorder in which diabetes is generally the first manifestation to occur, we hypothesized that homozygosity mapping could be a powerful genetic tool to identify candidates for *EIF2AK3* sequencing among infants with isolated permanent neonatal diabetes (PNDM) and reported parental consanguinity.

We report the successful use of homozygosity mapping for early molecular diagnosis of WRS. In addition, we describe the clinical and genetic findings in the largest international cohort of WRS cases assembled to date.

MATERIALS AND METHODS

This study was conducted in accordance with the Declaration of Helsinki and informed consent was obtained from all patients, with parental consent given on behalf of children.

Study population

All patients were referred to the Exeter Molecular Genetics Laboratory (UK) for testing. Clinical information was obtained using a standardized questionnaire from hospital records. Parental consanguinity was defined as parents being 2nd cousins or closer. A total of 34 probands were tested for *EIF2AK3* mutations. These included 28

probands with a suspected diagnosis of WRS on the basis of early-onset diabetes (within the first 15 months of age) and either skeletal dysplasia and/or unexplained liver dysfunction, and six consanguineous probands with isolated PNDM at referral in whom the most common genetic causes of diabetes had been previously excluded (including *KCNJ11* and *ABCC8* in all of them, *INS* in 5, and *GCK* in 4) and a large (9.22–67.64 Mb) region of homozygosity encompassing the *EIF2AK3* gene on chromosome 2 had been identified (see below).

Homozygosity mapping

Genotyping was carried out on the Affymetrix human 10K Xba and 50K Hind mapping SNP chips by Medical Solutions Nottingham (formerly GeneService), UK, or the Affymetrix 5.0 mapping chip by ALMAC Diagnostics Carigavon, Northern Ireland. Processing of genomic DNA was performed as per the Affymetrix protocol and the mean SNP call rate was 98.7%. In house Perl scripts were developed to automatically identify genomic homozygous segments for the 10K chip, defined by at least 20 consecutive homozygous SNPs marking a region that exceeded 3 cM (27). These thresholds were empirically extended to the larger chips identifying any region >3 Mb delimited by consecutive homozygous SNP calls, allowing for a maximum of 2 heterozygous SNPs per 100 calls. All regions for each case were assigned a rank, in descending size order.

***EIF2AK3* gene analysis**

Genomic DNA was extracted from peripheral leukocytes using standard procedures. The coding exons and the intron-exon boundaries of the *EIF2AK3* gene were PCR-amplified; primers and conditions are available upon request. Sequence specific primers for each amplicon were tagged with 5' M13 tails to allow sequencing to be performed with a "universal" M13 primer. Single strand sequencing was carried out using standard methods on an ABI 3730 (Applied Biosystems, Warrington, UK). Sequences were compared to the published template (accession number AF110146.1) using Mutation Surveyor v3.20 (SoftGenetics, PA, USA). Any changes in the sequence were

checked against published polymorphisms and mutations and for conservation across species. Sequence variants were tested for their presence in family members whenever a DNA sample was available.

Further molecular testing in probands with *EIF2AK3* mutations

In case of proband 3021-1, we used a panel of microsatellites for chromosome 20 (D20S482, D20S851, D20S477, D20S107, D20S481, D20S171) to confirm family relationships. An alternate set of exon 11 primers were also designed to amplify across the original set of exon 11 specific primers, to exclude allelic drop out. We then designed a Multiplex Ligation-dependent Probe Amplification (MLPA) assay to quantify the number of copies of *EIF2AK3*. We used synthetic oligonucleotide probes for *EIF2AK3* exons 10-12 with *HNF1A* and *HNF4A* as control probes (method previously described by Ellard et al. (28)). To investigate uniparental isodisomy, a panel of microsatellite markers flanking *EIF2AK3* on chromosome 2p11.2-q11.2 (D2S2368, D2S139, D2S2333, D2S388, D2S2216, D2S2181, D2S2154, D2S113 and D2S2264) was used. The same set of microsatellite markers was also used to explore relatedness between probands 3377-1 and 3750-1.

Other genetic testing in patients from consanguineous pedigrees

The common genetic causes of PNDM were tested in 591 patients diagnosed with diabetes within the first 6 months after birth. *KCNJ11* (NM_000525), *ABCC8* (NM_000352.2), and *INS* (NM_000207) were screened in all of the patients. *GCK* (NM_000162.2) testing was limited to those patients born to consanguineous parents in whom a homozygous region encompassing the gene on chromosome 7 had been identified using the mapping chips (see above). Genetic analysis was performed as described above for *EIF2AK3*.

Statistical analysis

Clinical numeric data is given as median and interquartile range (IQR). Standard deviation scores (SDS) for birth weights were calculated by comparing to the Child Growth Foundation LMS data (29). The clinical features of the patients were analyzed using Kruskal-Wallis and Mann Whitney-U tests, and Spearman correlation coefficient in the statistical package SPSS version 15.0 (Chicago, USA). χ^2 test was used to compare the frequencies of the different genetic subtypes of PNDM in consanguineous and non-consanguineous pedigrees.

RESULTS

Molecular genetic findings

We identified 26 different *EIF2AK3* mutations in 25 probands (Table 1). Figure 1 displays the pedigrees of the 5 families with more than one affected individual. Twenty-three probands had a homozygous mutation and two were heterozygous for two different mutations. Six mutations had previously been described and 20 mutations were novel, including nonsense (n=8), frameshift (n=7), missense (n=4), and splicing (n=1) mutations. Twenty-three probands had private mutations. The same homozygous mutation (R587X) was present in two probands from Turkey and the possibility of a founder effect could not be excluded by a combination of intragenic SNP and microsatellite analysis.

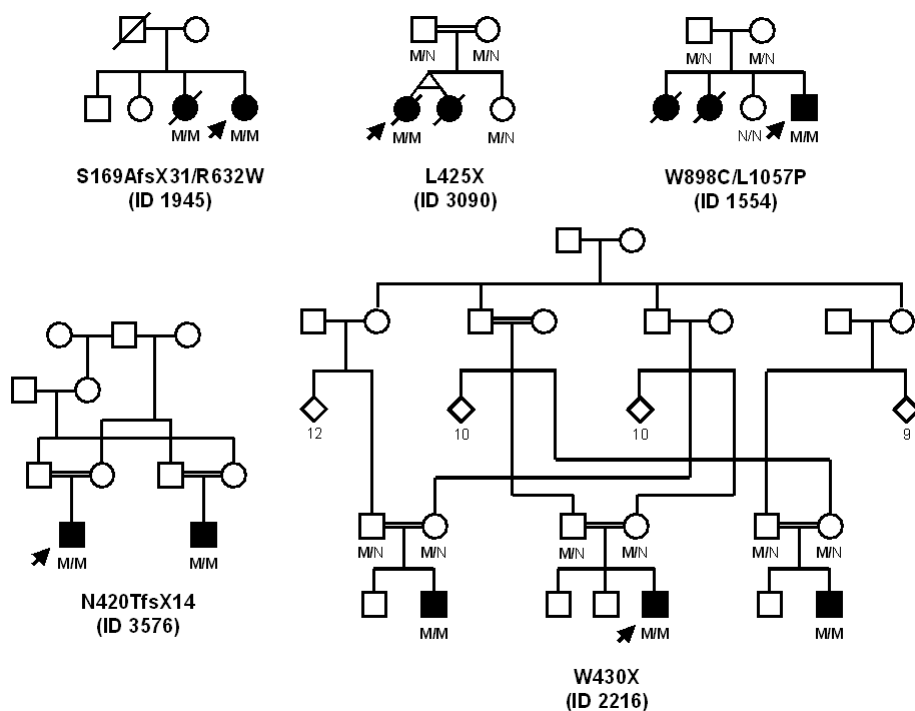


Figure 1. Pedigrees for families with more than one affected individual. Solid black filled shapes represent patients with Wolcott-Rallison syndrome. An arrow indicates the proband. The genotype is shown underneath each symbol; M and N denote mutant and normal alleles, respectively. ID: family identification number.

Proband ID	Reported parental consanguinity	Country of origin	EIF2AK3 mutation (nucleotide)	EIF2AK3 mutation (protein)	Mutation reported previously
1305-1	Yes	Turkey	c.2153delG	S718TfsX6	No
1554-1 ¹	No	Slovakia	c.2694G>T, c.3170T>C	W898C/ L1057P	Ref. 9
1945-1	No	UK	c.1894C>T, c.505delA	R632W/ S169AfsX31	No
2190-1 ²	Yes	Qatar	c.1567_1570del	E523X	Ref. 8
2216-1	Yes	UAE	c.1290G>A	W430X	No
2556-1	Yes	Morocco	c.2953G>A	G985R	No
3377-1	No	Turkey	c.1759C>T	R587X	No
3395-1	Yes	UAE	c.2867G>A	G956E	Ref. 31
3576-1	Yes	Saudi Arabia	c.1259delA	N420TfsX14	No
3822-1	Yes	Saudi Arabia	c.3190C>T	R1064X	No
3926-1	Yes	Morocco	c.449delA	K150RfsX2	No
4064-1	No	Saudi Arabia	c.475delG	A159PfsX41	No
4191-1	No	USA	c.1544_1559del	V515GfsX5	No
1229-1	Yes	Pakistan	c.935C>A	S312X	No
1537-1	Yes	Turkey	c.2981+1dupG	IVS14+1dupG	No
2660-1	No	Germany	c.2704C>T	R902X	No
2930-1	Yes	UAE	c.1949T>C	I650T	No
3021-1	No	UK	c.1774T>C	F592L	No
3090-1	Yes	India	c.1274T>A	L425X	No
3169-1	Yes	Libya	c.1032dupT	K345X	Ref. 1
3172-1	Yes	Saudi Arabia	c.1044_1057del	V349SfsX3	No
3750-1	No	Turkey	c.1759C>T	R587X	No
2045-1	Yes	Saudi Arabia	c.1406C>G	S469X	No
3547-1	Yes	India	c.2304_2305del	C768X	No
3705-1	Yes	Turkey	c.1562G>A	W521X	Ref. 9

UK: United Kingdom; UAE: United Arab Emirates.

¹ Patient previously reported by Senée et al (Ref. 9).

² Patient previously reported by Engelmann et al (Ref. 30).

Table 1. Mutation data.

The predicted effect of the different mutations on the EIF2AK3 protein is shown in Figure 2. The nonsense and frameshift mutations were distributed throughout the gene.

They are predicted to result in truncated proteins missing all or part of the catalytic domain and hence expected to lead to a complete loss of function. Pathogenicity of the 4 novel missense variants (F592L, R632W, I650T and G985R) was suggested by a) homozygosity or compound heterozygosity for the variant in affected individuals within families, b) no unaffected family members were homozygous or compound heterozygotes for the variant(s), c) location in one of the two serine/threonine protein kinase domains, and d) conservation of the amino-acid residues involved across species (including chimpanzee, rat, mouse, dog, chicken, Tetraodon, fruitfly, and *C. elegans*). One patient was homozygous for an intronic variant that changes the splice donor site of exon 14 from GTGAG to GGTGAG and is likely to be pathogenic, either by a direct effect on splicing leading to exon skipping or retention or by incorporating an extra base in exon 14 that would result in a frameshift mutation.

DNA was available from the parents of 16 probands. All unaffected parents were heterozygous carriers except for the father of 3021-1. The unaffected mother was heterozygous for the F592L mutation. Microsatellite marker analysis confirmed family relationships (results not shown). To determine the genetic mechanism of disease we excluded allelic-drop out (due to a SNP under the original primers) by re-sequencing exon 11 with an alternate set of specific primers. A paternally-inherited heterozygous deletion was also excluded by MLPA (data not shown). Further analysis using microsatellites flanking *EIF2AK3* (chr2p13.3-2q11.2) showed segmental maternal uniparental isodisomy for a minimal ~446Kb region encompassing the *EIF2AK3* gene (Figure 3).

No mutations in *EIF2AK3* were identified in six patients with a suggestive phenotype (3 with early-onset diabetes and skeletal dysplasia, and 3 with diabetes and liver dysfunction; none of them from consanguineous descent) and in further 3 consanguineous probands with isolated PNDM.

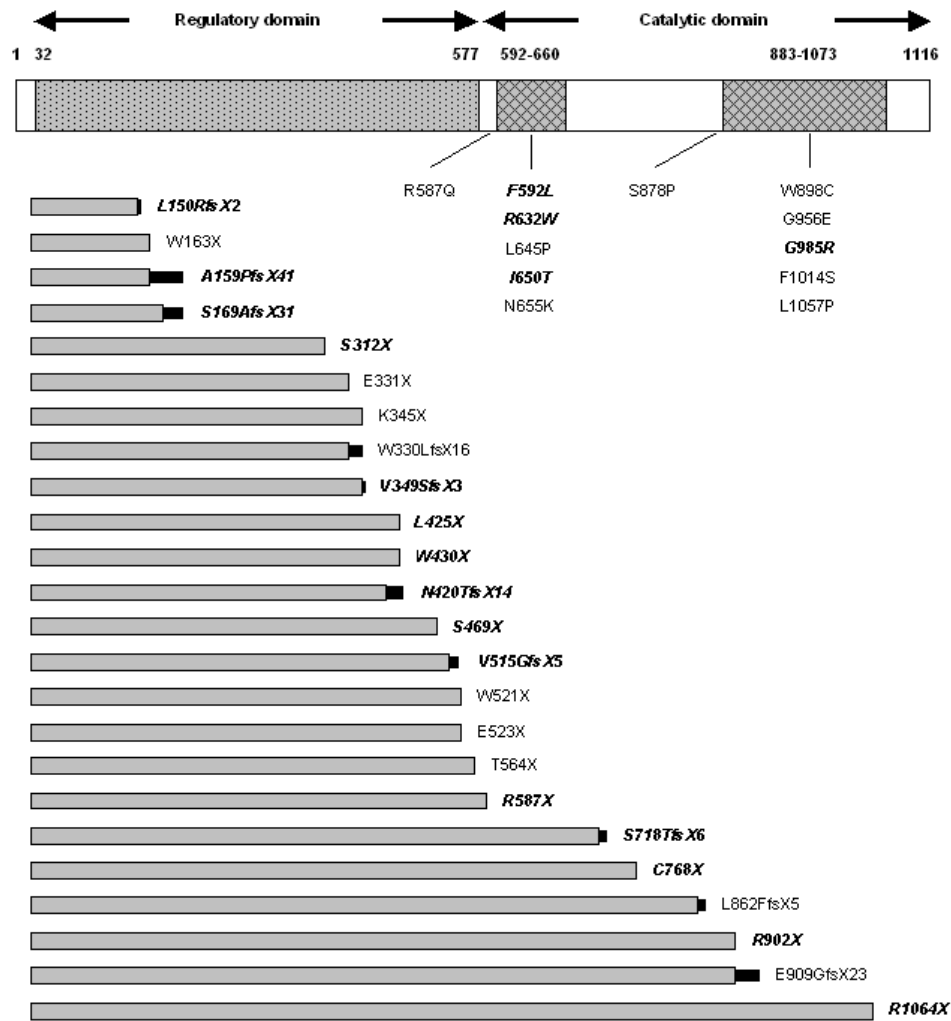
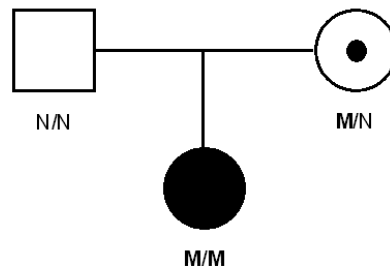


Figure 2. Summary of the effect of all the mutations identified to date on the EIF2AK3 protein (modified from reference 9). The structure of the EIF2AK3 protein is illustrated in the upper part of the chart, with the regulatory (dotted bar) and the two conserved serine/threonine protein kinase subdomains (squared bars) in the catalytic domain indicated. Novel mutations identified in this study are depicted in italics. Missense mutations are grouped directly under the region involved. The bars in the lower part of the chart indicate the extent of the EIF2AK3 mutant proteins, with the black portion representing abnormal amino acid sequence secondary to frameshift mutations. Further three other *EIF2AK3* mutations have been identified, including two splicing mutations (c.2981+1G>A and c.2981+1dupG, the latter in the present study) and a 184-bp deletion in exon15/intron 15.



Locus	Cytogenetic location	Father		Proband		Mother		
D2S2368	2p13.3	84	102	102	88	88	88	Heterozygous
D2S139	2p12	178	194	194	188	188	172	Heterozygous
D2S2333	2p12	258	256	256	256	256	254	Non informative
D2S388	2p11.2	258	264	264	255	255	260	Heterozygous
D2S2216	2p11.2	148	148	146	146	146	146	Homozygous (isodisomy)
D2S2181	2p11.2	272	272	272	272	272	272	Non informative
<i>EIF2AK3</i>	<i>2p11.2</i>	<i>N</i>	<i>N</i>	<i>F592L</i>	<i>F592L</i>	<i>F592L</i>	<i>N</i>	
D2S2154	2q11.1	180	180	180	180	180	180	Non informative
D2S113	2q11.2	254	220	220	208	208	222	Heterozygous
D2S2264	2q11.2	254	252	252	258	258	254	Heterozygous

Figure 3. **Microsatellite markers analysis in family 3021.** The solid black filled circle represents the proband with Wolcott-Rallison syndrome. The genotype is shown underneath each symbol; M and N denote mutant and normal alleles, respectively. The table shows genotype analyses of family members using 9 microsatellite markers around *EIF2AK3* on chromosome 2; the disease-relevant mutation is shown in italics. Apparent homozygosity for the informative marker D2S2216 in the proband indicated maternal segmental isodisomy for chromosome 2 (bold).

Clinical features

Twenty-two of the 25 probands with biallelic mutations in *EIF2AK3* were selected for genetic testing because of a clinical phenotype suggesting WRS. The remaining 3 were tested following the identification of a large homozygous region in chromosome 2 encompassing the *EIF2AK3* gene (Table 2, Supplementary Table 1). At the time of this study, three probands had died aged between 7 and 14 months (a further affected relative was deceased at 4 years of age due to an unrelated condition, Supplementary Table 1). The median age of the remaining ones was 4.95 years (range: 1.2 - 32.3). The phenotype of the parents and heterozygous siblings was unremarkable.

	N=29
Age at diagnosis of diabetes (weeks)	10.5 (6.0 – 20.3)
Age at referral for testing (years)	3.3 (1.3 – 7.6)
Current age (years, n=25)	4.8 (2.8 – 7.8)
Age at death (years, n=4)	0.6 – 4
Males (%)	48.3
Birth weight (g)	2805 (2515 – 3173)
Birth weight (SDS)	-1.39 (-2.08 – -0.59)
Skeletal dysplasia at referral (%)	48.3
Liver dysfunction at referral (%)	72.4

Table 2. Clinical features at time of referral for patients with a proven genetic diagnosis. Data is generally given as median (IQR). Age at death is given as full range.

All probands first presented with permanent diabetes mellitus, at a median age of 10.5 weeks (IQR: 6.0 - 19.3). The earliest presentation was at 3 weeks and all patients but one were diagnosed within or slightly after the first 6 months of life. The only patient diagnosed after 1 year of age (14 months) is homozygous for a missense I650T mutation. There are no differences in age at diabetes onset among the different types of mutation ($p=0.46$). Although patients with WRS have a reduced birth weight (median: -1.4 SDS, IQR: -2.1 to -0.6), intrauterine growth retardation defined as a birth weight at or below -2 SDS was present only in 7 of 20 patients for whom that information was available. There was no significant correlation between birth weight and age at diagnosis of diabetes ($p=0.33$, $p=0.16$). All patients are currently on full-replacement insulin doses, suggesting endogenous insulin secretion is negligible.

In addition to diabetes, skeletal abnormalities (mostly spondyloepiphyseal dysplasia) were initially reported by the referring clinician in probands from 13 families making the clinical diagnosis of WRS likely. Probands with skeletal abnormalities at referral did not differ from patients without in terms of either birth weight ($p=0.73$) or age at diabetes onset ($p=0.87$). However, they were significantly older at molecular testing

[median age: 6.6 years (IQR: 4.0-10.8) vs. 1.4 years (IQR: 0.9-1.8), $p=0.02$]. Nine further probands were tested because of the association of early-onset diabetes and liver dysfunction ranging from mild hypertransaminasemia to acute liver failure requiring a transplant. Although none of them presented clinically evident skeletal abnormalities at referral, they have become evident after molecular diagnosis in at least 2 cases. Major hepatic dysfunction was often accompanied by acute multiorgan failure (encephalopathy, renal failure, bone marrow failure) and sometimes resulted in the patient's death. Hepatic and renal functions usually returned to normal in patients who survived. Residual cerebellar signs (ataxia, dysarthria) were found in two patients after recovery from an acute encephalopathic episode. The oldest patient in the series, currently aged 32 years, is the only one having chronic renal failure and erythropoietin-dependent anemia; the remaining patients are currently 15 years old or younger. Pancreatic exocrine insufficiency requiring supplemental enzymes was present in two patients, with severe pancreatic hypoplasia reported in one of them.

Three consanguineous patients with isolated PNDM received a molecular diagnosis between 0.8 and 1.6 years following the identification of a large homozygous region encompassing *EIF2AK3*. One of them, currently aged 1.6 years, has not presented any other features of WRS as yet. Another one was found to have hepatomegaly and irregular fragmented epiphyses shortly after the molecular diagnosis was made at 1.2 years. The third one has developed the full clinical picture of WRS by the age of 3 years, including liver dysfunction identified at 1 year and skeletal abnormalities at 2 years. Interestingly, he was also diagnosed with primary hypothyroidism at 1.4 years but this may not be related to the *EIF2AK3* mutation.

Known parental consanguinity was reported in 17 of the 25 families; affected individuals from all of them have a homozygous *EIF2AK3* mutation. Two probands were compound heterozygotes for two different mutations and a third was homozygous for an *EIF2AK3* mutation as a result of segmental uniparental isodisomy of chromosome 2. The remaining 5 probands were homozygous for an *EIF2AK3* mutation. Three originate from

countries with high rates of consanguinity (Turkey and Saudi Arabia) (32) and the other two are from relatively isolated populations (Kosovo and South Dakota, USA).

Prevalence of Wolcott-Rallison syndrome among patients with PNDM

Mutations in *EIF2AK3* account for 15 of 63 (23.8%) consanguineous probands with PNDM tested in the Exeter laboratory. WRS is the most common known genetic cause of PNDM in consanguineous pedigrees, followed by recessive mutations in *INS* (12.7%), *GCK* (11.1%), and *ABCC8* (6.3%). This is in contrast to non-consanguineous families, where WRS accounts for only 8 of 583 cases (1.4%, $p=7 \times 10^{-20}$). Heterozygous mutations in *KCNJ11*, *ABCC8* and *INS*, the most common genetic causes of PNDM in non-consanguineous pedigrees (36.9%), are responsible for only 4.8% of PNDM cases in consanguineous families ($p=3 \times 10^{-7}$).

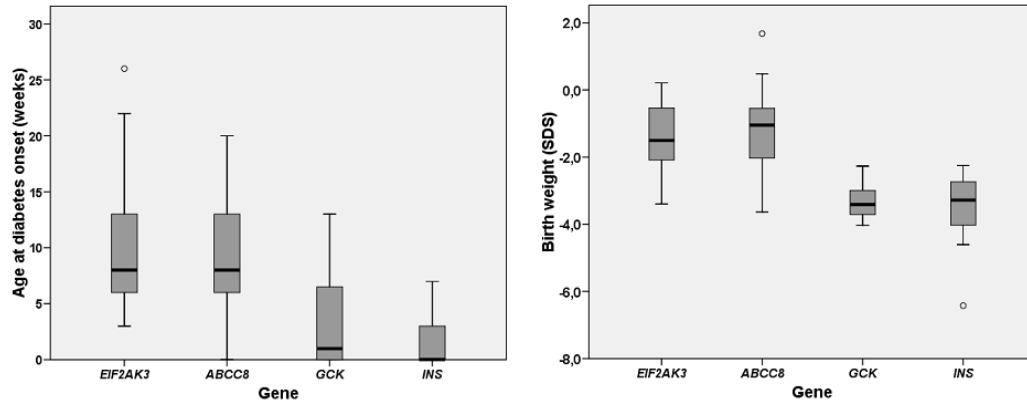


Figure 4. Age at diabetes onset (panel A) and adjusted birth weight (panel B) in the four most common recessive genetic causes of PNDM. There is no significant difference in age at presentation between *EIF2AK3* and *ABCC8* ($p=0.60$). However, patients with *EIF2AK3* mutations are diagnosed with diabetes later than those with *INS* or *GCK* mutations ($p=0.001$ and $p=0.009$, respectively). Similarly, birth weight in *EIF2AK3* mutation carriers was slightly reduced and comparable to that of patients with a mutation in *ABCC8* ($p=0.63$) but significantly higher than birth weight of patients with recessive *INS* and *GCK* mutations ($p=0.001$ and $p=0.001$, respectively).

We compared the age at diagnosis of diabetes and birth weight of the 29 patients with *EIF2AK3* mutations with the three other most frequent forms of recessive PNDM from the Exeter cohort, including 18 patients with *ABCC8* mutations, 14 with *INS* mutations, and 8 with *GCK* mutations (Figure 4). There was a strong association of the genotype with both age at diagnosis of diabetes ($p=0.000005$) and birth weight adjusted by gestational age ($p=0.000001$). Patients with *EIF2AK3* mutations were not different from patients with recessive *ABCC8* mutations either in terms of age at diabetes onset or birth weight. However, they showed less severe intrauterine growth retardation and were diagnosed later than patients with recessive *INS* or *GCK* mutations.

DISCUSSION

We report the largest series of WRS assembled to date. Overall, a molecular diagnosis of WRS has been confirmed in 25 families. Twenty of the 26 mutations (77%) identified are novel. Most cases were selected for genetic testing following diagnosis of skeletal dysplasia and/or unexplained liver dysfunction in a patient with a previous diagnosis of neonatal or infancy-onset diabetes. However, a homozygosity mapping approach allowed the identification of three patients before any other abnormalities apart from diabetes became evident.

Genetic testing for mutations in *EIF2AK3* is usually delayed until the full clinical picture of Wolcott-Rallison syndrome is evident (9). As each intercurrent illness can potentially be complicated by acute liver and/or renal failure, this diagnostic delay might be responsible for the poor outcome of some patients who develop unexplained acute multi-organ failure during minor intercurrent illnesses and may explain that WRS syndrome may go unnoticed when the evolution is rapidly fatal before the skeletal involvement is evident (6, 33). Early diagnosis of WRS is important because it allows the anticipation of potential complications during concomitant situations such as acute illness, trauma, or major surgery (34). For this purpose, radiological screening for epiphyseal

abnormalities in any infant with diabetes has been recommended (34). However, this approach would lead to a high number of unnecessary X-ray surveys in infants with diabetes, among whom WRS is still a relatively rare condition. Moreover, in most spondyloepiphyseal or multiple epiphyseal dysplasias the bone lesions are discovered only after the first year of life, and sometimes even later (6), which argues against the potential benefit of a radiological screening method for WRS. Homozygosity mapping has been previously used for positional cloning of unknown genes producing a recessive disorder, but this is the first time it has been used for early diagnosis of a recessive disease before the full clinical picture is present.

In contrast to nonsense and frameshift mutations spread throughout the gene, missense mutations have only been identified within or nearby each of the two Ser/Thr protein kinase domains of the catalytic domain. This underscores the important functional role of these domains. Missense variants in other parts of the gene might be either very well tolerated or contribute to a less severe phenotype with later onset diabetes or milder skeletal abnormalities. In keeping with this possibility, Senée et al. (9) reported a patient with a homozygous N655K mutation in *EIF2AK3* who presented with diabetes at a relatively late age (2.5 yr). Functional studies showed that the mutation is hypomorphic so that the mutated protein still had some residual kinase activity. We identified a proband homozygous for an I650T mutation who also had a late onset of diabetes at 14 months. However, this patient developed acute liver failure requiring liver transplantation at 2 years, so a later onset of diabetes clearly does not predict a milder phenotype of the disease. Furthermore, skeletal abnormalities are not evident in our patient with the F592L mutation even though she is 32 years old and has developed many of the acute complications of WRS. The severity of the different clinical manifestations of WRS is largely independent of the genotype.

The majority of probands (88%) were homozygous for *EIF2AK3* mutations and were from known consanguineous pedigrees, isolated populations or countries where consanguinity is frequent (32). We report the first case of uniparental isodisomy for an *EIF2AK3* mutation in a patient who inherited the mutation only from her mother. This

emphasizes the importance of testing parents of patients with recessive disorders in order to offer an accurate genetic counseling as the risk of recurrence is almost negligible in this case. However, lack of reported consanguinity should not be used as an exclusion criterion for *EIF2AK3* testing in the presence of a typical clinical presentation as genetic mechanisms other than homozygosity may be encountered in patients with WRS.

We have shown that birth weight and age at diabetes onset can discriminate between *EIF2AK3* and other genetic subtypes of neonatal diabetes in consanguineous probands. Whilst patients with WRS usually have a normal or mildly reduced birth weight and are diagnosed with diabetes after the first 3 weeks of life, patients born with severe intrauterine growth retardation (birth weight below -2 SDS for gestational age) or diagnosed with diabetes within the first 3 weeks of life are more likely to have biallelic *INS* or *GCK* mutations.

In summary, *EIF2AK3* mutations are the most common cause of PNDM in consanguineous pedigrees. Besides testing patients with a definite clinical diagnosis of Wolcott-Rallison syndrome, we recommend that analysis of the *EIF2AK3* gene is considered in patients with isolated neonatal diabetes diagnosed after 3 weeks of age from known consanguineous pedigrees, isolated populations or countries where inbreeding is frequent.

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Patient ID	Reported parental consanguinity	Country of origin	Gender	Birth weight (g)	Gestational age (weeks)	Age at diabetes onset (wks)	Age at molecular diagnosis (yr)	Current age (or age at death, yr)	Skeletal dysplasia at testing	Liver dysfunction at testing	Other significant clinical features	EIF2AK3 mutation
1305-1	Yes	Turkey	Male	3250	40	19	3.4	5.1	Yes	No	Anemia, skin pigmentation.	S718TfsX8 (c.2153delG)
1554-1	No	Slovakia	Male	3750	42	12	10.9	14.2	Yes	No	Growth retardation and developmental delay. Two affected sisters died at 1 and 4 yr (acute liver failure during minor infections).	W898C/L1057P (c.2894G>T, c.3170T>C)
1945-1	No	UK	Female	4190	Term	26	11.8	14.5	Yes	Yes	Acute liver failure, encephalopathy, renal dysfunction, anemia, and thrombocytopenia during a minor febrile illness at 3 yr. Complete recovery except for residual cerebellar signs (ataxia, dysarthria) and mild learning disabilities. Growth failure, acro-osteolysis and multiple epiphyseal dysplasia. Os Odontoideum with spinal cord compression.	R632W/ S169AfsX31 (c.1894C>T, c.505delA)
1945-2	No	UK	Female	NA	NA	12	Post-mortem	Died at 4 yr	Yes	Yes	Down's syndrome. Acute multi-organ failure (liver, kidney, bone marrow) and encephalopathy during a viral respiratory infection at 3 yrs. Acro-osteolysis. Death from chemotherapy-related complications during treatment for myelodysplasia.	R632W/ S169AfsX31 (c.1894C>T, c.505delA)
2190-1	Yes	Qatar	Male	2800	Term	28	9.2	11.7	Yes	Yes	Glucose-6-phosphatase deficiency. Acute cholestatic liver failure and renal dysfunction. Failure to thrive, hyperlordosis, and evolving features of skeletal dysplasia. Os Odontoideum.	E523X (c.1567_1570del)
2216-1	Yes	UAE	Male	2800	40	7	4.3	5.8	Yes	Yes	Mild anemia. Abnormal LFT's at 3 yr. X-rays show some sort of epiphyseal dysplasia, delayed speech.	W430X (c.1290G>A)

Supplementary Table 1. Detailed clinical and genetic information of patients with a molecular genetic diagnosis of Wolcott-Rallison syndrome. NA: not available.

Patient ID	Reported parental consanguinity	Country of origin	Gender	Birth weight (g)	Gestational age (weeks)	Age at diabetes onset (wks)	Age at molecular diagnosis (yr)	Current age (or age at death, yr)	Skeletal dysplasia at testing	Liver dysfunction at testing	Other significant clinical features	EIF2AK3 mutation
2216-4	Yes	UAE	Male	2500	40	8	1.3	2.8	No	No	Pre Dx: Microcephaly, intermittent neutropenia at 6 weeks. Post Dx: hepatomegaly and recurrent hepatitis at 2 yr, chronic anemia.	W430X (c.1290G>A)
2216-7	Yes	UAE	Male	NA	NA	NA	0.5	1.9	No	No	Pre Dx: None. Post Dx: hepatomegaly and recurrent hepatitis at 15 mo, microcephaly and developmental delay, neutropenia at 15 mo.	W430X (c.1290G>A)
2556-1	Yes	Morocco	Female	3000	38	22	13.4	15.1	Yes	No	Anemia.	G985R (c.2953G>A)
3377-1	No	Turkey	Female	3300	40	8	4	4.8	Yes	Yes	Growth failure, abnormal gait, hyperlordosis, spondyloepiphyseal dysplasia, bone pain. Pancreatic exocrine insufficiency (atrophic pancreas). Chronic liver dysfunction at 9 mo.	R587X (c.1759C>T)
3395-1	Yes	UAE	Male	2000	40	4	10.8	11.5	Yes	Yes	Spondyloepiphyseal dysplasia and osteopenia. Hepatomegaly and recurrent hepatitis. Microcephaly, gross motor delay. Transient renal dysfunction. Transient anemia. Pancreatic exocrine insufficiency.	G956E (c.2867G>A)
3576-1	Yes	Saudi Arabia	Male	2600	40	4	6.4	7.2	Yes	Yes	Recurrent liver dysfunction and hepatomegaly since 2.5 yr. Spondyloepiphyseal dysplasia at 6 yr.	N420TfsX14 (c.1259delA)
3576-2	Yes	Saudi Arabia	Male	2750	40	0	0.6	1.3	No	Yes	Pre Dx: Abnormal LFTs at 45 days. Skeletal survey not done as yet.	N420TfsX14 (c.1259delA)
3622-1	Yes	Saudi Arabia	Female	2800	40	20	6.6	6.6	Yes	Yes	Congenital nystagmus, microcephaly, hepatomegaly and recurrent hepatitis since 1 yr, neutropenia and anemia at 1 yr, spondyloepiphyseal dysplasia at 4 years.	R1064X (c.3190C>T)

Supplementary Table 1. Detailed clinical and genetic information of patients with a molecular genetic diagnosis of Wolcott-Rallison syndrome. NA: not available.

Patient ID	Reported parental consanguinity	Country of origin	Gender	Birth weight (g)	Gestational age (weeks)	Age at diabetes onset (wks)	Age at molecular diagnosis (yr)	Current age (or age at death, yr)	Skeletal dysplasia at testing	Liver dysfunction at testing	Other significant clinical features	EIF2AK3 mutation
3926-1	Yes	Morocco	Female	2800	39	21	3.3	3.6	Yes	Yes	Growth retardation, mild mental retardation. Hepatomegaly and recurrent cholestatic hepatitis. Skeletal abnormalities noted at 3 yr. Transiently impaired renal function. Transient neutropenia and anemia.	K150RfsX2 (c.449delA)
4064-1	No	Saudi Arabia	Male	3300	NA	6	7.6	7.8	Yes	Yes	Hepatitis and neutropenia at 3 yr.	A159PfsX41 (c.475delG)
4191-1	No	USA	Female	3093	NA	6	3.2	3.2	Yes	Yes	Skeletal abnormalities, transient abnormal LFTs	V515GfsX5 (c.1544_1559del)
1229-1	Yes	Pakistan	Female	2810	42	3	1.6	4.8	No	Yes	Hepatomegaly, liver enzyme derangement, anemia, failure to thrive.	S312X (c.935C>A)
1537-1	Yes	Turkey	Male	3200	40	4	0.9	Died at 0.9 yr	No	Yes	Recurrent episodes of cholestatic hepatitis. Death from multiorgan failure following upper respiratory tract infection.	IVS14+1dupG (c.2981+1dupG)
2660-1	No	Germany	Male	3250	39	12	1.5	2.2	No	Yes	Elevated LFTs during intercurrent illness. Post Dx: skeletal dysplasia at 18 months.	R902X (c.2704C>T)
2930-1	Yes	UAE	Male	3000	40	14 months	2.3	3.1	No	Yes	Neutropenia and anemia, gross motor delay, acute liver failure (transplant at 2 yr). Post Dx: short stature and abnormal gait (skeletal survey: marked skeletal dysplasia).	I650T (c.1949T>C)
3021-1	No	UK	Female	2600	39	12	31.9	32.3	No	Yes	Pre Dx: Multiorgan failure (liver, kidney) and encephalitis following respiratory tract infection at 2.5 yr. Complete recovery except from cerebellar ataxia and learning disabilities. Acute renal failure at 5 yr. Chronic renal failure (eGFR stable at 40ml/min).	F592L (c.1774T>C)

Supplementary Table 1. Detailed clinical and genetic information of patients with a molecular genetic diagnosis of Wolcott-Rallison syndrome. NA: not available.

Patient ID	Reported parental consanguinity	Country of origin	Gender	Birth weight (g)	Gestational age (weeks)	Age at diabetes onset (wks)	Age at molecular diagnosis (yr)	Current age (or age at death, yr)	Skeletal dysplasia at testing	Liver dysfunction at testing	Other significant clinical features	EIF2AK3 mutation
3090-1	Yes	India	Female	1500	40	6	1.1	Died at 14 months	No	Yes	Pre Dx: transient jaundice at 10-11 months (?hepatitis). Died at 14 mo (vomiting, diarrhea). Co-twin died at 6 weeks from unexplained disease.	L425X (c.1274T>A)
3169-1	Yes	Libya	Female	2200	40	9	4.7	4.8	No	Yes	Hepatomegaly and chronic elevated transaminases at 1 yr, mild motor delay, neutropenia at 2 years.	K345X (c.1032dupT)
3172-1	Yes	Saudi Arabia	Female	2900	NA	13	0.8	1.8	No	Yes	Pre Dx: abnormal LFTs at diabetes presentation.	V349SfsX3 (c.1044_1057del)
3750-1	No	Turkey	Female	2560	41	9	0.6	Died at 7 months	No	Yes	Pre Dx: Death from acute liver and renal failure following an episode of gastroenteritis.	R587X (c.1759C>T)
2045-1	Yes	Saudi Arabia	Male	2000	NA	4	0.8	3	No	No	Pre Dx: None. Post DX: hepatomegaly and chronic liver dysfunction at 1 yr, primary hypothyroidism at 1.3 yr (TSH: 146.6 (0.35-4.94) mU/L, FT4 <5 (9-19) pmol/L), spondyloepiphyseal dysplasia at 2 yr.	S469X (c.1406C>G)
3547-1	Yes	India	Female	NA	NA	13	1.6	1.6	No	No	Pre Dx: None.	C768X (c.2304_2305del)
3705-1	Yes	Turkey	Female	2500	40	22	1.2	1.4	No	No	Pre Dx: None. Post Dx: Hepatomegaly (5 cm), irregular fragmented epiphyses on skeletal survey.	W521X (c.1562G>A)

Supplementary Table 1. Detailed clinical and genetic information of patients with a molecular genetic diagnosis of Wolcott-Rallison syndrome. NA: not available.

CHAPTER 6

Recessive mutations in the *INS* gene result in neonatal diabetes through reduced insulin biosynthesis

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ABSTRACT

Heterozygous coding mutations in the *INS* gene that encodes preproinsulin were recently shown to be an important cause of permanent neonatal diabetes. These dominantly acting mutations prevent normal folding of proinsulin which leads to β -cell death through ER stress and apoptosis. We now report 10 different recessive *INS* mutations in 15 probands with neonatal diabetes. Functional studies showed that recessive mutations resulted in diabetes due to decreased insulin biosynthesis through distinct mechanisms including gene deletion, lack of the translation initiation signal, and altered mRNA stability due to the disruption of a polyadenylation signal. A subset of recessive mutations caused abnormal *INS* transcription, including the deletion of the C1 and E1 *cis* regulatory elements, or 3 different single base-pair substitutions in a CC dinucleotide sequence which was so far not known to be essential for *INS* promoter activity. In keeping with an earlier and more severe β -cell defect, patients with recessive *INS* mutations had a lower birth weight (-3.2 SDS vs. -2.0 SDS) and were diagnosed earlier (median 1 week vs. 10 weeks) compared to those with dominant *INS* mutations. Mutations in the insulin gene can therefore result in neonatal diabetes as a result of two contrasting pathogenic mechanisms. Moreover the recessively inherited mutations provide a genetic demonstration of the essential role of multiple elements that regulate the biosynthesis of insulin in man.

INTRODUCTION

Neonatal diabetes is diagnosed within the first 6 months of life (1, 2) and there are two main clinical subtypes: the persistent, permanent neonatal diabetes (PNDM) and the remitting and frequently relapsing, transient neonatal diabetes (TNDM). Recently there have been considerable advances in the understanding of the genetics of neonatal diabetes (3). Most patients with PNDM have activating mutations in *KCNJ11* or *ABCC8*, the genes encoding the potassium ATP-sensitive (K_{ATP}) channel subunits Kir6.2 (4) and SUR1 (5-7), or heterozygous mutations in the *preproinsulin* (*INS*) gene (8-12). In contrast, abnormalities in chromosome 6q24 are the most common cause of TNDM (13), followed by mutations in the *KCNJ11* and *ABCC8* genes (14). Despite these advances the etiology of neonatal diabetes is still not known in at least 30% of patients with PNDM, suggesting other genetic causes are still to be found (9).

Insulin is secreted from islet beta cells of the pancreas. Insulin biosynthesis and secretion are tightly regulated to maintain blood glucose levels within a narrow physiological range. Insufficient secretion of insulin results in hyperglycemia and diabetes, whereas excessive secretion results in hypoglycemia. The synthesis of insulin is tightly regulated. Extensive studies have dissected an array of *cis* sequence elements in the *INS* promoter region and their cognate DNA binding factors, which together ensure the cellular specificity and rate of *INS* transcription (15-22). In addition, insulin biosynthesis is strongly dependent on posttranscriptional regulatory mechanisms, including the modulation of translation and mRNA stability (23-25). The latter is largely mediated through sequences located in the untranslated regions of *INS* transcripts (26-28).

Heterozygous missense mutations in the coding region of the *INS* gene have recently been described as a cause of neonatal diabetes (8-12). Most of the reported mutations are predicted to disrupt the folding of the proinsulin molecule. The resulting misfolded protein accumulates in the endoplasmic reticulum (ER), resulting in ER stress

and beta cell apoptosis (29, 30). An alternative potential genetic mechanism would be reduced insulin secretion due to a disruption of the *INS* coding sequence, as seen in the double *Ins1* and *Ins2* knockout mouse (31), or of the sequences that regulate insulin biosynthesis. However, this has so far not been demonstrated in humans.

We report, for the first time, recessively acting mutations within the *INS* gene in a series of patients with neonatal diabetes. In contrast to the previously described dominant mutations, these mutations reduce insulin synthesis and thus represent a novel pathogenic mechanism for human diabetes. These mutations also provide genetic evidence for the essential role of distinct nucleotide sequences in the regulation of the human *preproinsulin* gene.

MATERIAL AND METHODS

Cohort characteristics

We studied an international cohort of 122 unrelated patients (69 males) with diabetes diagnosed before 6 months (median age 4 weeks) and without a known genetic etiology, which were referred to the Exeter (n=110) or Bilbao laboratories (n=12). Thirteen patients were offspring of consanguineous parents (2nd degree relatives or closer). In the 101 probands with PNDM we excluded mutations in *KCNJ11*, *ABCC8*, *GCK* and previously described heterozygous coding mutations in *INS* (9). In the 21 patients with TNDM we excluded 6q24 anomalies, *KCNJ11* and *ABCC8* mutations. Babies born before 33 weeks of gestation were excluded in order to avoid hyperglycemia of prematurity. Studies were approved by local ethics committees. Informed consent was obtained from all patients or their parents and the studies were conducted in line with the Declaration of Helsinki. Clinical data was obtained from the patients' clinical records. We calculated standard deviation scores (SDS) for birth weight (32).

Molecular genetic analysis

Genomic DNA was extracted from peripheral leukocytes using standard procedures. Regulatory elements up to 450 bp upstream of the transcriptional start site and exons 1-3 of the *INS* gene (Figure 1) were amplified by the polymerase chain reaction (PCR) in three amplicons (primers and conditions available on request). Unidirectional sequencing was carried out on an ABI3730 (Applied Biosystems, Warrington, UK) and analyzed using Mutation Surveyor v3.20. Sequences were compared to the published sequence (Ensembl sequence ENSG00000129965) and published polymorphisms. The genomic reference sequence nucleotide 1 is the transcriptional start site (g.1A or c.-238A) whilst the translational start site is located at g.238 (c.1). Mutation nomenclature is shown in compliance with HGVS, where nucleotide 1 represents the A of the translational start site codon ATG (c.1). Suspected mutations were tested for conservation across species and co-segregation within families. Putative gene deletions were investigated using Multiplex Ligation dependent Probe Amplification (MLPA) assay oligonucleotide probes specific for the three exons of *INS* (see Supplementary Methods).

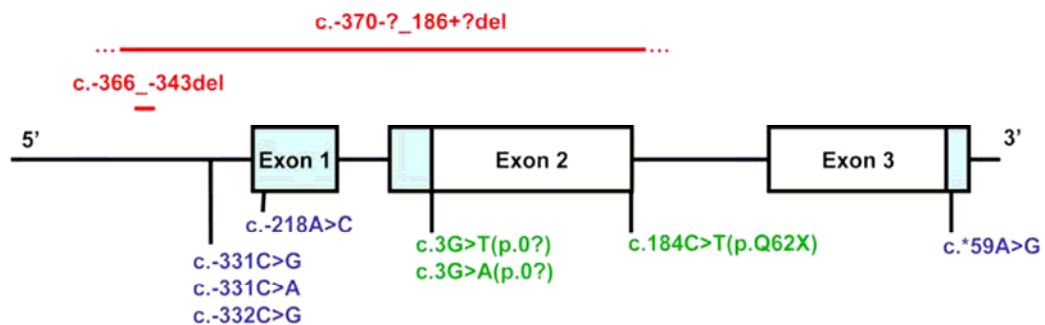


Figure 1. A schematic of the *INS* gene showing the 10 mutations identified in 15 families. Positions of point mutations are indicated below the exons, whilst deletions are shown above the gene. The blue shaded regions are non-coding, the red text indicates a deletion, the blue text are non-coding mutations and the green are coding mutations. The precise breakpoints of the multiexonic deletion are not known; the solid line represents the minimal deleted region. Mutation nomenclature is based on the coding sequence where nucleotide 1 represents translational start site.

Functional studies

Investigating the effect of INS promoter mutations on transcriptional activity

To determine the functional impact of the c.-331(C>G, C>A) and c.-332C>G mutations we performed site-directed mutagenesis of an *INS* promoter firefly luciferase reporter construct (251hINS-Luc), and compared the activity of control and mutated promoters in MIN6 β -cells and control cell lines, using a Renilla luciferase minimal promoter (pGL4.75) (see Supplementary Methods).

Investigating the effect of the translation initiation mutations (c.3G>T and c.3G>A)

To determine the effect of these mutations on insulin production we transfected HeLa cells, which do not express insulin, with wild type or mutant *INS* and analysed intracellular insulin content using radio-immunoassay (see Supplementary Methods).

*Investigating the effect of the c.*59A>G mutation on mRNA stability*

We determined the effect of the c.*59A>G mutation on insulin mRNA stability using real time-PCR to measure the relative levels of the *INS* mRNA transcripts in a heterozygous lymphoblastoid cell line derived from the proband's mother. We used a heterozygous SNP, rs3842753, to identify the mutation bearing allele (see Supplementary Methods).

Statistical analysis

Clinical numeric data is given as median (interquartile range). Functional data is given as mean (standard error). The clinical features of patients were compared using Kruskal Wallis, χ^2 (Fisher's exact) or Mann Whitney-U tests in the statistical package SPSS version 13 (Chicago, USA). Student's t-test or analysis of variance was used for expression studies.

RESULTS

Recessive *INS* mutations cause neonatal diabetes

We sequenced 122 unrelated probands with diabetes diagnosed before 6 months (13 offspring of consanguineous parents) in whom the known common genetic causes had been excluded. We identified 10 different *INS* recessive mutations in 15 unrelated families (Figures 1 and 2). Nine of the 15 pedigrees are known to be consanguineous as parents were second cousins or closer. Four homozygous mutations affected the coding region: c.184C>T (p.Q62X), c.3G>T (p.0?), c.3G>A (p.0?), and a large deletion that removes a segment of the promoter, exon 1 and coding exon 2 of *INS* (c.-370-?_186+?del). Five homozygous mutations were found in regulatory regions: c.-331C>A (2 families), c.-331C>G (5 families), c.-218A>C, and a 24 base pair deletion (c.-366_-343del) are located in the promoter region, whereas c.*59A>G is within the 3' untranslated region. One proband was a compound heterozygote for two regulatory region mutations, c.-331C>G and c.-332C>G.

The mutations were inherited in a recessive manner either homozygous or compound heterozygous with heterozygous carrier parents being unaffected (Figure 2). In keeping with the recessive inheritance, 9 of the 15 probands are born to consanguineous parents. Pathogenicity of mutations was suggested by conservation across species and absence of variants in controls (see Supplementary results).

Recessive *INS* mutations uncover essential regulatory sequences in man

Further support for the pathogenicity of mutations came from known function of mutated residues and functional studies (Figure 3, Figure 4 and Supplementary results). Multiple mutation mechanisms were involved in the recessive *INS* mutations which are described briefly below:

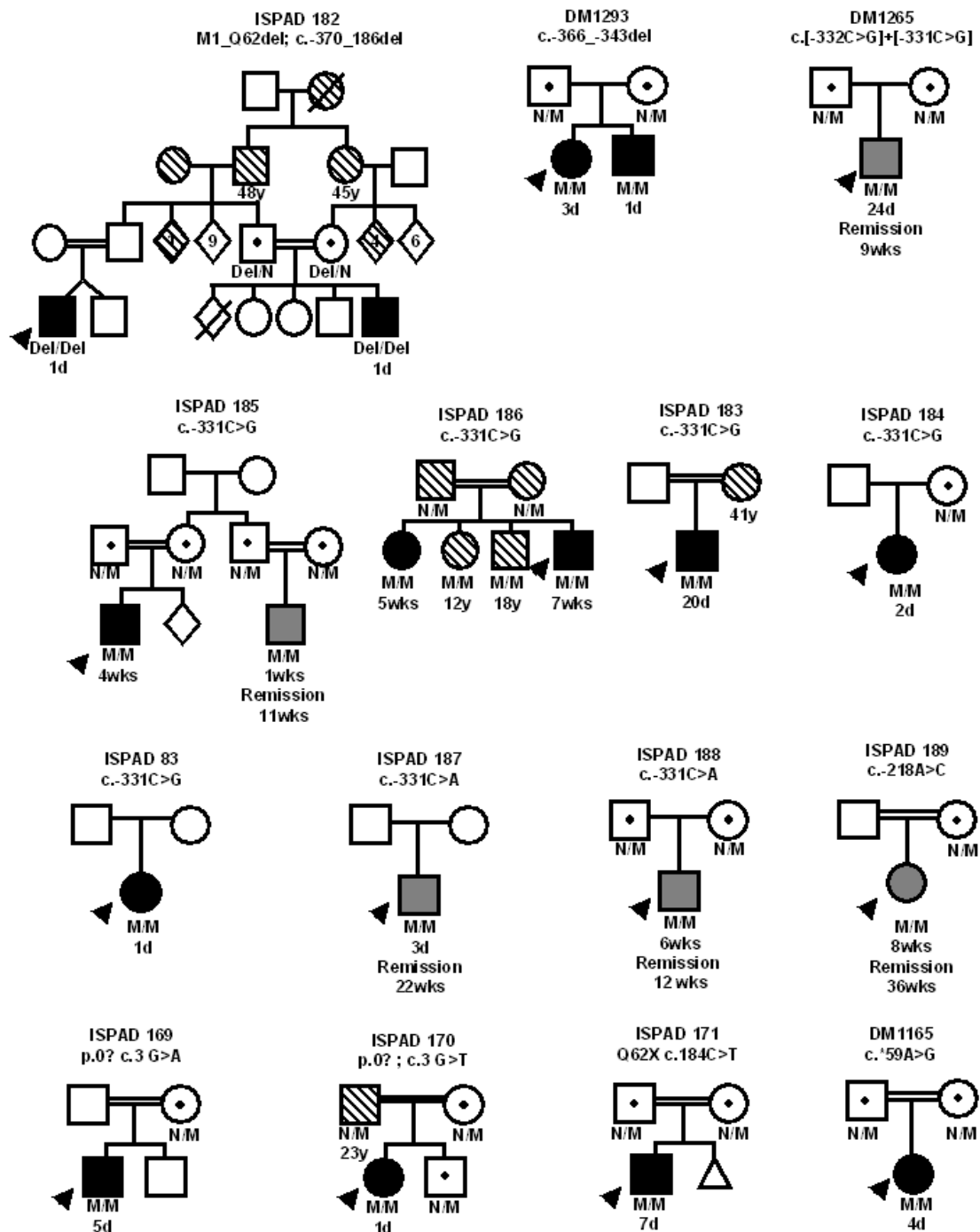


Figure 2. Partial pedigrees of the 15 families with recessive *INS* mutations. (N=Normal allele, M=Mutation, Del=Deletion). Solid black filled shapes represent patients with permanent neonatal diabetes, gray filled shapes represent patients with transient neonatal diabetes and shapes filled with diagonal lines represent those patients diagnosed with diabetes after 6 months of age. Age at diagnosis and remission (where applicable) is shown below the symbols.

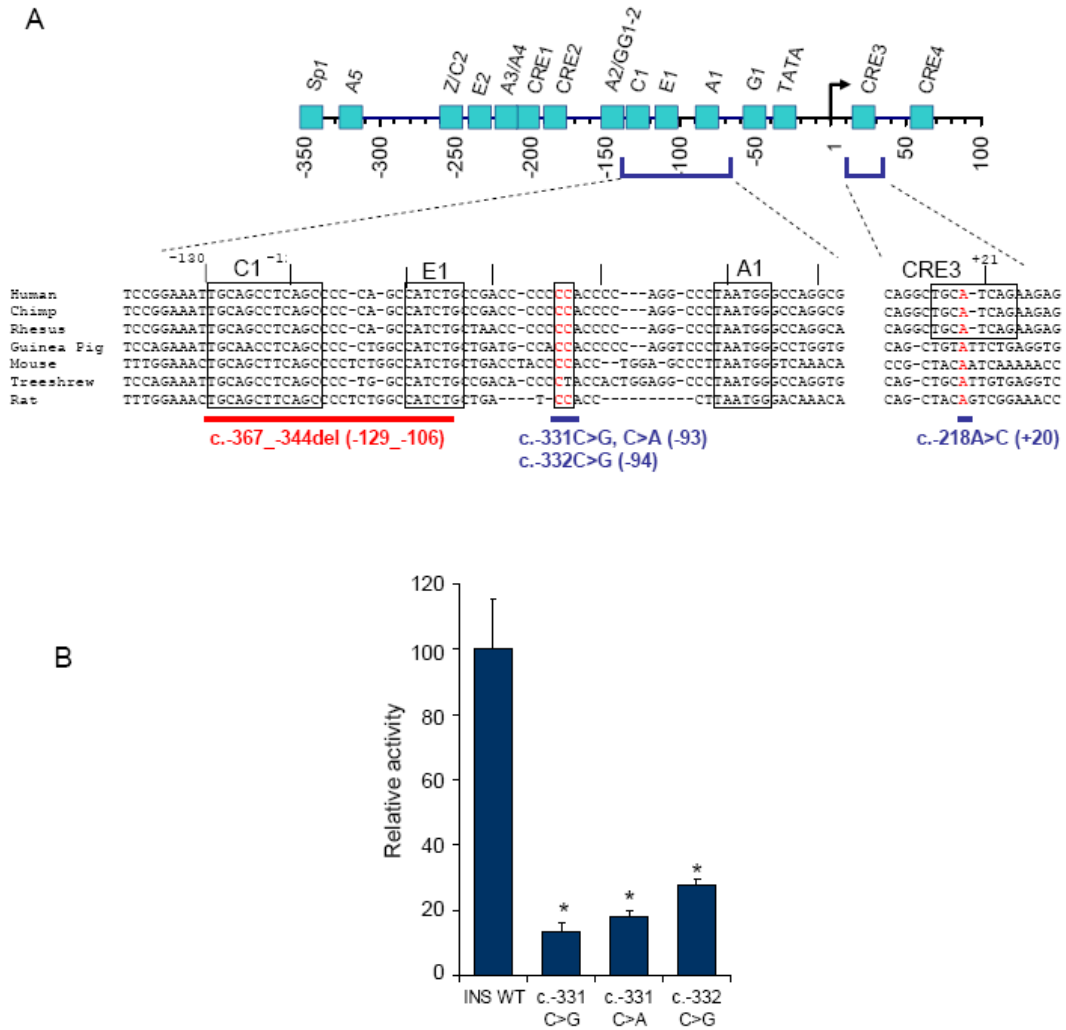


Figure 3. Functional evidence for the pathogenicity of recessive promoter *INS* mutations. (a) Schematic of the genomic sequence of the *INS* promoter structure with major *cis* regulatory elements, and the sequence context of mutated elements in several mammalian species that do not exhibit major divergence in these regions. Mutated bases are highlighted in red. The numbering of promoter landmarks is relative to the transcription start site (genomic numbering, where g.1 is equivalent to c.-238) consistent with the convention used in previous studies. Mutations are described according to HGVS guidelines (<http://www.hgvs.org/mutnomen/>) (cDNA numbering according to the translational start site where c.1 is equivalent to g.238), and distance to the transcription start site is shown in parenthesis. (b) Evidence for loss-of-function of the c.-331(C>G, C>A) and c.-332C>G mutations. Firefly luciferase expression is compared in constructs containing the wild type (WT) *INS* promoter sequence (*INS* WT), or c.-331 C>G, c.-331 C>A, c.-332 C>G mutations, after transfection in MIN6 β -cells. Data shown are means (+SE) from three independent constructs for each mutation, each performed in triplicate. Results are corrected for transfection efficiency using a vector that constitutively expresses Renilla luciferase, and expressed relative to the *INS* WT results. The asterisks denote $P<0.001$ in ANOVA for the difference between *INS* WT and mutant constructs.

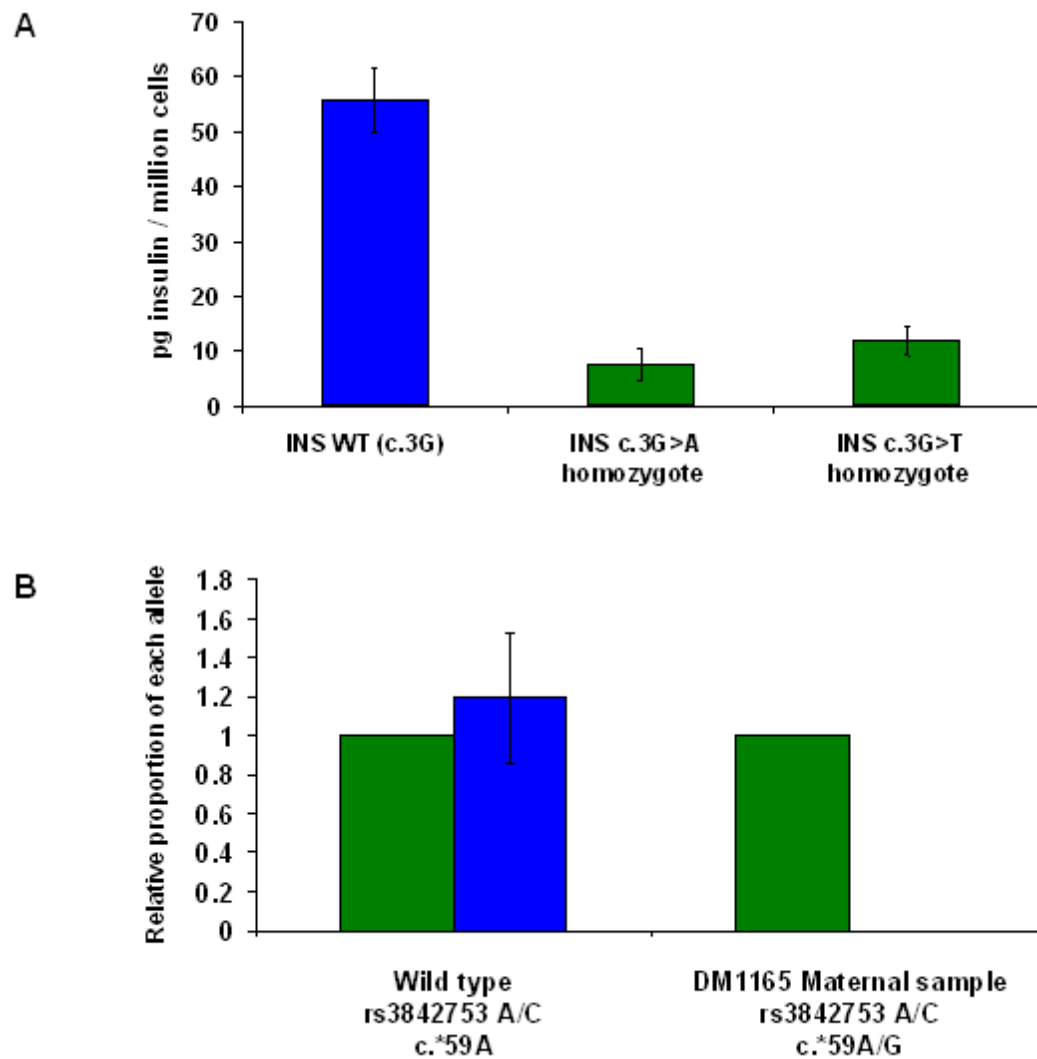


Figure 4. Functional evidence for the pathogenicity of recessive *INS* mutations affecting translation and mRNA stability. (a) Homozygous mutations in the translation initiation codon of the *INS* gene result in reduced insulin content of transfected HeLa cells. The insulin content of HeLa cells was measured by radioimmunoassay after transfection with wild type insulin (*INS* WT) or either of two *INS* mutant constructs, as shown. Both nucleotide changes were identified in patients with permanent neonatal diabetes. Non-specific values obtained with HeLa cells transfected with empty vector were subtracted from all samples and those data are presented as mean \pm SE ($n=3$ replicates). (b) Allele-specific quantitative real-time PCR of c.*59A>G and normal transcripts. The graph shows the relative abundance of the wild type and mutant RNA transcripts in mutant and normal cell lines. The rs3842753 A allele tags the c.*59A (wild type, shown in green), whilst the c.*59G (mutant) was tagged by rs3842753 C allele (blue). The graph shows the level of transcripts in the control sample heterozygous only for rs3842753 and in the maternal sample (family DM1165) which is heterozygous for both rs3842753 and c.*59A/G. The level of the mutant transcript is reduced to less than 3×10^{-4} per cent compared to the normal transcript in the heterozygous c.*59A>G cell line. Experimental error as calculated from the standard deviation (SD) of the replicate experiments is indicated. The SD for the quantification of the c.*59G allele in the maternal sample is 3×10^{-6} , and thus the experimental error is not visible in the figure.

- Truncated proteins: The nonsense mutation (p.Q62X) is predicted to give rise to a mutant protein that is truncated within the C peptide region and will lack the insulin A chain.
- Promoter mutations: The (c.-366_-343del) 24 base pair deletion abolishes the *INS* promoter evolutionary conserved C1 and E1 elements, where MAFA and NEUROD1 bind, respectively (16, 20, 33) (Figure 3A). The c.-218A>C mutation disrupts the CRE3 site that interacts with multiple DNA binding proteins *in vitro* (22) (Figure 3A). All of these elements have been previously shown to be critically important for the *INS* promoter activity in transient transfection studies (15, 18, 34-36). The c.-331(C>G, C>A) and c.-332C>G mutations were located in a more poorly characterized segment of the *INS* promoter situated between the E1 and A1 elements (Figure 3A). This sequence is conserved amongst a subset of mammalian species (Figure 3A). No interacting protein complex has been reported in binding studies that examined this region (17, 37), although a 3 base pair mutation that includes the 3' cytosine of this dinucleotide and two other nucleotides located further 3' partially impairs insulin promoter activity (37). We constructed insulin promoter fragments carrying the c.-331(C>G, C>A) and c.-332C>G mutations and showed up to 90% reduction in transcriptional activity (Figure 3B) in pancreatic beta-cell lines. Thus, the CC dinucleotide that is mutated in 8 unrelated probands with neonatal diabetes forms part of a positive *cis* regulatory sequence of the *INS* promoter.
- Mutated or absent translational start site: The two point mutations (c.3G>A and c.3G>T) at the first methionine residue (p.Met1) abolish the native translation initiation site for the preproinsulin protein. Quantification of total *INS* mRNA levels by real-time PCR revealed no differences in mRNA abundance for c.3G>A or c.3G>T mutations compared with the wild type. The insulin content of HeLa cells transfected with these mutations was reduced by 86% and 79% for c.3G>A and c.3G>T, respectively, compared to cells transfected with the wild type sequence (Figure 4A and Supplementary

results). The multi-exon deletion (exons 1 and 2) removes over half the coding region including the translational start site and is expected to be a null mutation.

- Altered mRNA stability through a mutation in the 3' untranslated region: The c.*59A>G mutation is located in the polyadenylation signal of the 3' untranslated region and potentially impairs mRNA stability. In a heterozygous lymphoblastoid cell line generated from the proband's mother the mutant mRNA transcript was present at a very low level compared to the wild type allele. This is consistent with reduced mRNA stability (see Figure 4B and Supplementary results).

Clinical phenotype of patients with recessive *INS* mutations

The clinical characteristics of patients with recessive *INS* mutations are shown in Table 1 (and Supplementary results). In keeping with the known actions of insulin before and after birth, the phenotype was limited to markedly reduced fetal growth and diabetes.

Characteristic	INS recessive	INS dominant	p-value
n	19	46	NA
Sex (% male)	63.2	47.8	0.3
Birth weight (g)	1680 (1410, 2050)	2530 (2350, 2900)	<0.001
Gestational age (wk)	37.5 (36, 40)	40 (38.5, 40)	0.008
Birth weight (SDS)	-3.2 (-4.1, -2.6)	-2.0 (-2.5, -1.0)	<0.001
Age at diagnosis (weeks)	1 (0, 3)	10 (5, 22)	<0.001
Remission (%)	26	0	0.001
Age at remission (wk)	12 (11, 22)	NA	NA
Age at relapse (yr)	1 (1 case)	NA	NA
Current age (yr)	5 (2, 14)	11 (4, 23)	0.2

Table 1. Comparison of clinical characteristics in patients with isolated neonatal diabetes with recessive and dominant *INS* mutations. Data are median (interquartile range). NA: not applicable.

The diabetes phenotype within the families is shown in Figure 2. Nineteen patients had neonatal diabetes (15 probands and 4 family members); 14 had PNDM and were treated with insulin from diagnosis, whilst 5 patients had TNDM having gone into

remission at a median age of 12 weeks (IQR 11, 22). Birth weight was markedly reduced in all patients with neonatal diabetes resulting from recessive mutations [median birth weight 1680 g (1420, 2050) which is -3.2 SDS (-4.1, -2.6)]. In keeping with more severe insulin deficiency, patients with PNDM had a more severe intrauterine growth retardation [median SDS for birth weight -3.9 (-4.4, -2.8) vs. -1.8 (-3.4, -0.9) in TNDM, $p=0.03$] and diabetes was diagnosed earlier [2 days (1, 9.5) vs. 24 days (5, 62), $p=0.04$] (Supplementary results). All patients with mutations that altered the coding region or mRNA stability had PNDM. The noncoding promoter mutations were associated with both PNDM and TNDM.

Differences in the clinical phenotype with recessive and dominant *INS* mutations

To identify if the different mutation mechanisms in the same gene resulted in phenotypic differences we compared the clinical characteristics of patients with neonatal diabetes due to recessive *INS* mutations with patients with the previously identified dominant mutations in *INS* (Table 1). Patients with neonatal diabetes resulting from recessive *INS* mutations had a markedly different phenotype with lower birth weight [median SDS score -3.2 (IQR -4.1, -2.6) vs. -2.0 (-2.5, -1.0), $p < 0.001$] and an earlier age of diagnosis [median age in weeks 1 (0, 3) vs. 10 (5, 22), $p < 0.001$]. TNDM is only seen in patients with recessive mutations (26 vs. 0%, $p=0.001$).

DISCUSSION

We have shown that recessively acting mutations in the *preproinsulin* gene (*INS*) are a novel cause of neonatal diabetes. They act by reducing synthesis of the preproinsulin peptide due to a truncated protein, abnormal transcription, reduced mRNA stability or disrupted translation. These mutations usually cause PNDM but may manifest as TNDM or diabetes outside the neonatal period. In keeping with the recessive inheritance, many probands (60%) were the offspring of consanguineous parents.

The clinical manifestations of recessive *INS* mutations reflect the consequences of insulin deficiency in humans during pre- and postnatal life. The birth weight was markedly reduced [median SDS score -3.2 (-4.1, -2.6)], consistent with the major role of insulin in fetal growth. The early onset of neonatal diabetes (median 1 week) reflects severe insulin deficiency postnatally. In contrast to many other subtypes of neonatal diabetes, there are no extrapancreatic features.

Differences in the underlying pathophysiology explain why patients with recessive *INS* mutations are diagnosed earlier and have a lower birth weight than patients with heterozygous *INS* mutations (8-12). The disrupted insulin synthesis seen with recessive mutations occurs as soon as the fetal beta cell starts to secrete insulin. In contrast insulin secretion is required before beta cell dysfunction develops in patients with heterozygous mutations which result in misfolding of the preproinsulin peptide, accumulation of the misfolded protein in the endoplasmic reticulum (ER) and hence the destruction of the beta-cell through ER stress. These two distinct disease mechanisms are supported by phenotypic studies in mouse models, where reduced insulin secretion at birth or progressive ER stress and cell death have been described in mice carrying analogous recessive or dominant mutations, respectively (30, 31, 38).

The majority of the patients with neonatal diabetes have PNDM, but 26% (5/19) have TNDM. TNDM is only found in patients with non-coding mutations and they have a higher birth weight and are diagnosed later. This is consistent with TNDM resulting from a less severe insulin deficiency, and is comparable to the situation with mutations in the Kir6.2 subunit of the *KATP* mutations (39) where TNDM mutations have less severe functional consequences. The mechanism of remission in recessive *INS* mutation carriers is not understood but is likely to reflect a variation in demand or the ability of the beta-cell to meet this demand as a similar timing of remission is seen in some patients with less severe mutations resulting in channelopathies (14, 39) and pancreatic developmental defects (40, 41).

The mutations identified in this study illustrate multiple mechanisms by which insulin biosynthesis can be disrupted. These include absent/altered translation due to coding sequence deletions or mutations, reduced transcription due to mutations of the promoter, or abnormal mRNA stability. Our functional studies established that the 3' UTR mutation that abolishes the polyadenylation signal results in severe RNA instability and that the initiation codon mutations result in reduced transcription of the *preproinsulin* gene. The promoter mutations are highly informative because they provide human genetic evidence that discrete *INS* *cis* regulatory elements are essential. Numerous studies have demonstrated that multiple *cis* elements are required for the activity of episomal *INS* reporter constructs in cultured cells (15, 17-19, 21, 22). However, it is not known if each of those *cis* elements is truly necessary *in vivo*, because such studies can only partially predict their function in the integrated chromatin environment of true differentiated cells. Studies in other selected genes have addressed this by targeted deletion of transcriptional regulatory elements in mice (42). We have now established for the first time that the C1/E1, CC, and CRE3 elements are essential for *INS* gene transcription in humans. The discovery of 3 separate mutations that target the CC dinucleotide sequence is particularly significant. One earlier study reported that an artificial 3 base pair mutation that disrupts one C nucleotide of this element leads to a partial decrease of *INS* promoter activity (37). However, the CC element or its surrounding sequence have not been thoroughly characterized, and it is not recognized as an essential regulatory element of the *INS* promoter (21, 22). The importance of the CC and CRE3 *cis* elements for insulin biosynthesis warrant the need to identify the DNA binding factors that act through these elements.

In conclusion we have shown that homozygous *INS* mutations are a novel cause of neonatal diabetes. The mutations result in reduced synthesis of the insulin peptide through a variety of mechanisms and may yield further insights into the regulation of insulin biosynthesis.

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SUPPLEMENTARY INFORMATION

Supplementary Methods

Gene dosage analysis using Multiplex Ligation dependent Probe Amplification (MLPA)

We designed MLPA assay oligonucleotide probes (supplementary table 1) to measure the number of copies of *INS* exons 1-3 using probes for *HNF1A* and *HNF4A* as controls (method previously described by (1).

Methodology for the investigation of the effect of promoter mutations on insulin gene promoter activity

A plasmid containing nucleotides -489 to -228 of the human insulin gene promoter linked to the firefly luciferase gene (Dr Roland Stein, Vanderbilt University) was used to perform site-directed mutagenesis with the QuickChange kit (Stratagene, Texas). This plasmid is named -251hINS-Luc plasmid because it includes nucleotides -251 to +10 according to a previous reference sequence (2). Three independent mutations were created for each base substitution. They were verified by direct sequencing, and assayed in transient transfection assays in triplicate on two separate occasions as described (3). Mouse 10T/2 fibroblasts and MIN6 β cells were trypsinized 14 hours before transfection, distributed into 12-well plates (3×10^4 cells/well) and maintained in DMEM supplemented with 15% FBS. Cells were transfected with promoter-luciferase or empty vector plasmid DNA (300 ng) using Lipofectamine 2000 (Invitrogen). pGL4.75[hRLuc/CMV] (10 ng) was added to control for transfection efficiency. Firefly and Renilla luciferase were assayed with the Dual-Luciferase Reporter Assay System (Promega), and the ratios were expressed relative to the wild type insulin promoter values.

Methodology for investigating the effect of the translation initiation codon mutations (c.3G>T and c.3G>A)

DNA encoding wild-type *INS* and both mutant forms of *INS*-p.0?,c.3G>T and c.3G>A, were cloned into pcDNA 3.1/*myc*-His A. Correct insertion was verified by sequencing of the plasmids. 4×10^6 HeLa cells were cotransfected with 8 μ g pcDNA 3.1 and 8 μ g pMAX GFP using Nucleofector technology (Amaxa/Lonza) according to the manufacturer's instructions. GFP expression was examined by fluorescence microscopy to monitor transfection efficiency. Control cells were transfected with empty pcDNA3.1 vector.

Non insulin expressing HeLa cells were cultured in DMEM Glutamax medium (Gibco Life Technologies, Paisley, UK) supplemented with 5% FBS, 100 units/ml penicillin and 100 μ g/ml streptomycin at 37°C in a fully humidified atmosphere of 5% CO₂.

To rule out an effect of these mutations on mRNA stability, we quantified the amount of *INS* mRNA relative to the Beta-2-microglobulin (*B2M*) gene in transfected cells. Total RNA was extracted from approximately 1×10^6 EBV-transformed lymphoblastoid cells) using the Perfect RNA Mini RNA kit (Eppendorf, Hamburg, Germany). 4.5 μ g mRNA was treated with 1u of RNase-free DNase (TURBO DNase kit, Ambion, UK) 30 minutes at 37°C followed by 85°C for 5 minutes for nuclease inactivation. Complementary DNA (cDNA) was synthesized from mRNA using the Thermoscript RT-PCR system (Life Technologies, Paisley, UK) with an incubation temperature of 50°C and a random hexamer primer. PCR products were detected by the use of a probe situated across the boundary of exons 2 and 3 to ensure amplification from cDNA rather than genomic DNA (forward primer; CGGGAGGCAGAGGACCT, reverse primer; AGGCTGCCTGCACCAG, probe; 6FAM- ACCTGCCCCACCTGC-MGB). Reactions contained 5 μ l TaqMan Fast Universal PCR Master Mix, no AmpErase™, 0.9 μ M each primer and 0.25 μ M probe in a total volume of 10 μ l on the ABI prism 7900HT platform (Applied Biosystems, Warrington, UK). Amplification conditions were a single cycle of 95°C for 20 seconds followed by 60 cycles of 95°C for 1 second and 60°C for 20 seconds. The results given are an average of quantifications from three replicate amplifications.

Expression levels of the mutant and wild type transcripts were measured by comparing the number of cycles at which the *INS* and *B2M* PCR products cross a specific threshold. The relative abundance is defined by the difference in number of cycles to achieve the same quantity of product and calculated using the equation $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is the difference between the crossing points ΔC_t^{test} in a test sample normalised to a reference sample (ΔC_t^{ref}) (Applied Biosystems, Foster City, USA (4)). Differences in the mRNA abundance for wild-type and mutant transcripts were examined for statistical significance by Kruskal Wallis test.

The insulin content of transfected HeLa cells was determined 24 hours post-transfection by radioimmunoassay. Briefly, cells were lysed in 200µl of acidified ethanol (0.8M HCl:98% ethanol mixed in a ratio of 1:3) for 2 hours at -20°C and samples then neutralized by addition of NaOH. In triplicate, 50µl of each experimental sample or crystalline recombinant human insulin standard was mixed with 50µl of ^{125}I insulin (Linco; diluted to yield approximately 2000 cpm/50µl) and 50µl Guinea Pig anti-bovine insulin antibody (ICN) diluted 1:20000 in insulin assay buffer (IAB; 150mM NaCl, 30mM Na_2HPO_4 , 10mM KH_2PO_4 , 10mM EDTA, 5mg/ml bovine serum albumin, pH 7.4). Tubes were incubated at 4°C overnight before addition of 50 µl of donkey anti-Guinea Pig coated cellulose beads (IDS Ltd, Tyne and Wear, UK) (diluted 1:1 with IAB). After incubation for 1h, 2ml dH_2O was added and tubes centrifuged for 5 minutes at 1000g. The supernatant was aspirated under vacuum and the radioactivity retained in each pellet measured on a WALLAC gamma counter. The insulin content of each sample was then calculated by reference to a standard curve constructed with recombinant human insulin.

*Methodology for investigating the effect of the c.*59A>G mutation on mRNA stability*

Cell lines were established from peripheral blood lymphocytes derived from the proband's heterozygous mother (DM1165) and an unaffected control by EBV transformation. Cell lines were maintained in 1X RPMI-1640 (Gibco Life Technologies, Paisley, UK), supplemented with 10% fetal calf serum (Gibco Life Technologies, Paisley, UK).

Total RNA was extracted from approximately 1×10^6 EBV-transformed lymphoblastoid cells) using the Perfect RNA Mini RNA kit (Eppendorf, Hamburg, Germany). 4.5 µg mRNA was treated with 1u of RNase-free DNase (TURBO DNase kit, Ambion, UK) 30 minutes at 37°C followed by 85°C for 5 minutes for nuclease inactivation. Complementary DNA (cDNA) was synthesized from mRNA using the Thermoscript RT-PCR system (Life Technologies, Paisley, UK) with an incubation temperature of 50°C and a random hexamer primer.

Ectopic mRNA transcripts were amplified from lymphoblastoid cells using a single tube TaqMan™ approach. It was not possible to design probes for the c.*59A>G mutation due to its location at the extreme 3' end of the transcript. Instead we utilized a heterozygous single nucleotide polymorphism (rs3842753) located 37 nucleotides upstream of the poly-A tail to differentiate the mutation-bearing and normal transcripts. Probe and primer sequences were designed to this variant and validated by standard curve analysis [Forward – 5' ggagaactactgcaactagac 3', Reverse – 5' catctctctcggtgcaggag 3', Probe (WT) – VIC-cagccccAcacccg-MGB, Probe (MT) – 6-FAM-agccccCcacccg-MGB].

2 µl cDNA from the proband's mother (heterozygous for c.*59A>G and rs3842753) was used for real-time PCR quantification and cDNA from a normal cell line (heterozygous for rs3842753 only). PCR products were detected by the use of mutation-specific probes which were identical except for the site of the mutation. Reactions contained 5 µl TaqMan Fast Universal PCR Master Mix, no AmpErase™, 0.36 µM each primer and 0.08 µM each probe in a total volume of 10 µl on the ABI prism 7900HT platform (Applied Biosystems, Warrington, UK). Amplification conditions were a single cycle of 95°C for 20 seconds followed by 60 cycles of 95°C for 1 second and 60°C for 20 seconds. The results given are an average of quantifications from three triplicate amplifications derived from two separate RNA extractions.

To validate the real time assay we conducted standard curve analysis which indicated that the assay was accurate and quantitative over seven serial 1:2 dilutions.

The efficiency of amplification as assessed by the gradient of the standard curves for mutant and wild-type probes were -3.1 and -3.0 respectively. The correlation between crossing point and input template (r^2) was 0.95 and 0.92 for the mutant and wild-type probes respectively. The A and C allele transcripts of rs3842753 are present in approximately equal amounts in the normal heterozygous cell line, indicating that the presence of this SNP does not adversely affect the stability of either allele.

Expression levels of the mutant and wild type transcripts were measured using the allele specific assays by comparing the number of cycles at which the two different PCR products cross a specific threshold. The relative abundance is defined by the difference in number of cycles to achieve the same quantity of product and calculated using the equation $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is the difference between the crossing points (ΔCt^{test}) in a test sample normalized to the difference between mutant and normal crossing points in a 50:50 mixture ($\Delta Ct^{50\%}$) (Applied Biosystems, Foster City, USA (4)).

Supplementary Results

Large deletion detected by MLPA

A large deletion encompassing the promoter region, the non-coding exon 1 and coding exon 2 of *INS* (c.-370-?_186+?del) was identified in a large consanguineous Lebanese family (ISPAD 182). The deletion was identified following failure of PCR amplification of *INS* exons 1 and 2. The MLPA assay showed that the parents had a 50% reduction in dosage for exons 1 and 2 but two copies of exon 3. Testing the proband and affected cousin showed that *INS* exon 1 and 2 MLPA probes did not bind to the target DNA (suggestive of a homozygous deletion).

The effect of the translation initiation codon mutations on insulin content

The homozygous mutations c.3G>T and c.3G>A were identified in two unrelated probands with permanent diabetes diagnosed on the first day of life. Both are non-

synonymous changes affecting the first methionine residue (p.Met1) and abolishing the translation initiation site for the preproinsulin protein. A second methionine residue is located at codon 5 (p.Met5), but the surrounding sequence does not conform to the Kozak consensus for translation since a thymine base is present at position -3 to the ATG codon rather than the required adenine (5). We would therefore predict that translation from the mutated initiator codon would be compromised but without experimental evidence we could not rule out a possible effect on mRNA stability.

To investigate the possibility that these p.0? mutations (c.3G>T and c.3G>A) could affect mRNA stability, we quantified the amount of *INS* mRNA in HeLa cells expressing wild-type or mutation bearing plasmids by real-time PCR. There were no differences in the amount of *INS* mRNA expressed in cells transfected with either mutation compared with cells transfected with wild-type plasmids (*INS* wild-type: *INS* c.3G>T and *INS* wild-type: *INS* c.3G>A ratios were 1:0.99 (SD=0.19) and 1:0.95 (SD=0.15) respectively; p=0.875).

In order to investigate the possibility of low levels of translation from p.Met5, we used an *in-vitro* radio-immuno assay to measured insulin content in cells expressing the mutations compared to cells expressing wild type (WT) *INS*. The cells expressing *INS* (WT) contained 56 pg of insulin per million cells whilst the insulin content was reduced by at least 79% in the cells expressing *INS* c.3G>T and c.3G>A (11.9 and 7.6 pg of insulin per million cells respectively) (Figure 3C). This data shows that p.Met5 is unlikely to reinitiate translation of preproinsulin in the presence of the c.3G>T or c.3G>A mutations.

*The effect of the c.*59A>G mutation on RNA stability*

To investigate the effect of the c.*59A>G mutation on RNA stability we used real time PCR to quantify the level of *INS* mRNA transcripts in a heterozygous lymphoblastoid cell line generated from the proband's mother (DM1165). We were unable to discriminate between the transcripts directly via the c.*59A/G alleles, as the mutation was located at the far 3' end of the transcript. Instead we used a SNP (rs3842753) to tag the mutation,

with the mutation (c.*59G) on the same haplotype as the C allele of the SNP. The mutation-bearing transcript was not detectable in the heterozygous mother (Figure 3D) and in the homozygous state we predict that the affected child will not express the *INS* gene.

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Recessive *INS* mutations in neonatal diabetes

Probe name and size in base pairs (bp)	Forward (5'-3'probe)	Reverse (5'-3'probe) with 5' phosphate
MLPA_INS_EXON1 (115bp)	<i>gggttcctaagggttgga</i> GAACAGACC TGCTTCATGGCCTCT	TCTGATGCAGCCTGTCCTGGAG <i>GGtcagaaatcatcaaattttatcaatctaga</i> <i>ttgatcttgctggcac</i>
MLPA_INS_EXON2 (125bp)	<i>gggttcctaagggttgga</i> GAGCTTCCA CCAGGTGTGAGCCGC	ACAGGTGTTGGTTCACAAAGGC TGaatcaaaactgaacttttgagaaattgatg <i>atattctagattggatcttgctggcac</i>
MLPA_INS_EXON3 (140bp)	<i>gggttcctaagggttgga</i> AATGCCACG CTTCTGCAGGGACCC	CTCCAGGGCCAAGGGCTGCAG GCTattcatgttgaaattttattgatctgttca <i>tttgcattgaagtactctagattggatcttgctg</i> <i>gcac</i>

Supplementary Table 1. Synthetic probes designed for *INS* MLPA assay. Text in lower case italics are PCR primers, text underlined are 'stuffer fragments' non sequence specific probes, text in capitals are sequence specific for the *INS* gene.

Variant	n	Conservation					Caucasian control chromosomes	Pakistani control chromosomes	Experimental evidence for loss of function
		Mouse	Dog	Horse	Lizard	Xenopus			
c.-331C>G or c.-331C>A	7	Yes	No (A)	No (G)	Not present	Not present	306		Yes
.c.-332C>G	1	Yes	Yes	No (T)	Not present	Not present	306		Yes
c.-218A>C	1	Yes	Yes	Yes	Not present	Not present	306		ND
*c.188-15G>A	1	No (A)	Not present	Not present	Not present	Not present		404	ND
c.*59A>G	1	Yes	Yes	Yes	Not present	Not present	200		Yes

ND = not done.

*This intronic variant is unlikely to be pathogenic since the nucleotide is not conserved throughout evolution (conserved only in Rhesus) and an effect on splicing is unlikely

Supplementary Table 2. Assessment of pathogenicity of non-coding genetic variants giving data on the conservation of the nucleotides and the number of control chromosome sequences tested. The variants were not detected in any of the control chromosomes.

Family identifier	Sex	Mutation	Relationship to proband	Ethnicity	Known consanguinity	Age at diag. (days)	Glucose at diag. (mmol/l)	Disease status	Age at remission/relapse (weeks)	Birth weight (Kg)	GA (weeks)	Current age (yrs)	Current treatment (U/Kg/d)	Current HbA1c
DM 1293	F	c.-366_-343del	Proband	Caucasian	No	3	13	PNDM	NA	1.45	41		Insulin	6.2
DM 1293	M	c.-366_-343del	Brother	Caucasian	No	1	14	PNDM	NA	1.5	36	14.2	Insulin	7.6
DM 1265	M	c.-332C>G + c.-331C>G	Proband	Caucasian	No	24	34	TNDM	9	2.5	37	1.9	None	4.8
ISPAD185	M	c.-331C>G	Proband	Arabic	Yes	28	20	PNDM	NA	1.8	40	4	Insulin (0.5)	NA
ISPAD185	M	c.-331C>G	Cousin	Arabic	Yes	7	18	TNDM	11	1.8	40	2	None	9.0
ISPAD186	M	c.-331C>G	Proband	Arabic	Yes	50	27	PNDM	NA	2.4	40	15	Insulin (1.0)	11.5
ISPAD186	F	c.-331C>G	Sister	Arabic	Yes	35	NA	PNDM	NA	NA	NA	25	Insulin	9.0
ISPAD186	F	c.-331C>G	Sister	Arabic	Yes	4380	NA	Diabetes	NA	NA	NA	22	Insulin	11.6
ISPAD186	M	c.-331C>G	Brother	Arabic	Yes	6570	10	Diabetes	NA	NA	NA	19	Insulin	14.0
ISPAD183	M	c.-331C>G	Proband	Turkish	Yes	20	28	PNDM	NA	1.5	40	21	Insulin (1.0)	8.5
ISPAD184	F	c.-331C>G	Proband	Caucasian	No	2	40	PNDM	NA	1.8	37	10	Insulin (0.6)	7.3
ISPAD 83	F	c.-331C>G	Proband	Caucasian	No	1	16	PNDM	NA	1.0	36	10	NA	7.1
ISPAD 187	M	c.-331C>A	Proband	Caucasian	No	3	29	TNDM	22	1.9	38	3.2	None	NA
ISPAD 188	M	c.-331C>A	Proband	Bangladeshi	No	42	15	TNDM	12	2.7	40	2	None	6.1
ISPAD 189	F	c.-218A>C	Proband	Indian	Yes	56	27	TNDM	36/48	2.6	40	1.1	Insulin (1.0)	NA
ISPAD 169	M	c.3G>A (p.0?)	Proband	Hispanic	Yes	5	28	PNDM	NA	1.8	37	1.7	Insulin (0.6)	5.6
ISPAD 170	F	c.3G>T (p.0?)	Proband	Pakistani	Yes	1	16	PNDM	NA	1.2	36	5.7	Insulin (0.9)	8.5
ISPAD 171	M	c.184C>T (p.Q62X)	Proband	Indian	Yes	7	59	PNDM	NA	1.3	35	0.8	Insulin (0.6)	8.3
DM1165	F	c.*59A>G	Proband	Caucasian	Yes	4	NA	PNDM	NA	1.5	39	27.5	Insulin	6.2
ISPAD 182	M	c.-370-?_186+?del	Proband	Lebanese	Yes	1	NA	PNDM	NA	1.3	35	3.9	Insulin	7.5
ISPAD 182	M	c.-370-?_186+?del	Cousin	Lebanese	Yes	1	1	PNDM	NA	1.6	36	8.9	NA	8.7

Supplementary Table 3. Clinical characteristics of the 21 patients with recessive *INS* mutations. GA Gestational age. NA: not applicable/available. All mutations are recessive except for DM1265 which is compound heterozygous for the 2 mutations (inherited in *trans*).

Characteristic	Transient	Permanent	p-value
n	5	14	NA
Type of mutation (% regulatory)	100	64	0.3
Sex (% male)	80	57	0.6
Birth weight (g)	2540 (1860, 2650)	1500 (1300, 1800)	0.007
Gestational age (wk)	38 (37.5, 40)	37 (36, 40)	0.3
Birth weight (SDS)	-1.8 (-3.4, -0.9)	-3.9 (-4.4, -2.8)	0.03
Age at diagnosis (days)	24 (5, 62)	2 (1, 9.5)	0.04
Glucose at diagnosis (mmol/L)	27 (18, 31)	22 (15, 31)	0.7
Bicarbonate at diagnosis (mmol/L)	9 (7, 12)	23 (20, 24)	0.06
Remission (%)	100	0	NA
Age at remission (wk)	12 (11, 22)	NA	NA
Age at relapse (yr)	1 (only 1 case)	NA	NA
Current age (yr)	2 (1.5, 3)	10 (4, 18)	0.02
Current HbA1c (%)	5.5 (4.8, 6.1)	8.3 (6.7, 8.9)	0.04

Supplementary Table 4. Comparison of clinical characteristics in patients with permanent or transient neonatal diabetes due to an *INS* mutation. Data are median (interquartile range). NA: not applicable.

Characteristic	INS recessive coding	INS recessive non coding	INS dominant	p-value
n	5	14	46	NA
Sex (% male)	80.0	57.1	47.8	0.4
Birth weight (g)	1310 (1240, 1695)	1800 (1500, 2470)	2530 (2325, 2900)	<0.0005
Gestational age (wk)	36 (35, 36.5)	39 (37, 40)	40 (38.5, 40)	0.2
Birth weight (SDS)	-3.3 (-3.7, -2.8)	-3.1 (-4.4, -2.2)	-2.0 (-2.5, -1.0)	0.003
Age at diagnosis (wk)	0 (0, 0.5)	1 (0, 4.5)	10 (5, 22)	<0.0005
Remission (%)	0	36	0	<0.0005
Age at remission (wk)	NA	12 (11, 22)	NA	NA
Age at relapse (yr)	NA	1 (only case)	NA	NA
Current age (yr)	5 (1, 6)	10 (2, 15)	11 (4, 23)	0.4

Supplementary Table 5. Comparison of clinical characteristics in patients with recessive coding, recessive non coding or dominant *INS* mutations. Data are median (interquartile range). NA: not applicable.

CHAPTER 7

Homozygous mutations in *NEUROD1* are responsible for a novel syndrome of permanent neonatal diabetes and neurological abnormalities

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Author contributions: O.R-C., J.A.L.M., and S.E. and A.T.H. designed research; O.R-C., J.A.L.M., I.K., and D.W. performed research; O. R-C. J.A.L.M., S.E., and A.T.H. analyzed data; and O.R-C, and A.T.H. wrote the paper.

ABSTRACT

Objective: *NEUROD1* is expressed in both the developing and the mature beta cells. Studies in mice suggest this basic helix–loop–helix transcription factor is critical in the development of endocrine cell lineage. Heterozygous mutations have previously been identified as a rare cause of maturity-onset diabetes of the young (MODY). We aimed to explore the potential contribution of *NEUROD1* mutations in patients with permanent neonatal diabetes.

Research Design and Methods: We sequenced the *NEUROD1* gene in 44 unrelated patients with permanent neonatal diabetes of unknown genetic etiology.

Results: Two homozygous mutations in *NEUROD1* (c.427_428del and c.364dupG) were identified in two patients. Both mutations introduced a frameshift which would be predicted to generate a truncated protein completely lacking the activating domain. Both patients had permanent diabetes diagnosed in the first 2 months of life with no evidence of exocrine pancreatic dysfunction and a morphologically normal pancreas on abdominal imaging. In addition to diabetes, they had learning difficulties, severe cerebellar hypoplasia, profound sensorineural deafness, and visual impairment due to severe myopia and retinal dystrophy.

Conclusions: We describe a novel clinical syndrome that results from homozygous loss of function mutations in *NEUROD1*. It is characterized by permanent neonatal diabetes and a consistent pattern of neurological abnormalities including cerebellar hypoplasia, sensorineural deafness, and visual impairment. This syndrome highlights the critical role of *NEUROD1* in the development of the endocrine pancreas and central nervous system in humans.

INTRODUCTION

Monogenic permanent neonatal diabetes (PNDM) is typically diagnosed within the first six months after birth in contrast to polygenic autoimmune type 1 diabetes which is usually diagnosed later in childhood or in young adults (1, 2). PNDM is both phenotypically and genetically heterogeneous. Most patients present with isolated diabetes, but in some cases diabetes appears in the context of a more complex multi-systemic syndrome. Dominant mutations in three genes (*KCNJ11*, *ABCC8* and *INS*) are the cause of PNDM in more than 50% of cases and in the majority diabetes is an isolated finding (3, 4). Recessive mutations, autosomal or X-linked, have been described in 10 genes (*ABCC8*, *GCK*, *EIF2AK3*, *FOXP3*, *IPF1*, *PTF1A*, *GLIS3*, *SLC2A2*, *SCL19A2*, and *WFS1*). These are rare and often result in extra-pancreatic features in addition to neonatal diabetes (3). The genetic cause remains unknown in approximately 40% of patients with PNDM (Hattersley & Ellard, unpublished data).

From a pathogenetic perspective, a number of different mechanisms can lead to PNDM. Firstly, beta cells may be present but not functional as in patients with activating mutations in *KCNJ11* and *ABCC8*, the genes encoding the two subunits of the ATP-sensitive potassium channel genes (Kir6.2 and SUR1, respectively). Secondly, the number of beta cells may be reduced due to an increased destruction, either by apoptosis (*INS*, *EIF2AK3*) or as a consequence of an autoimmune insult (*FOXP3*). Finally there may be a reduced number of beta cells as a result of impaired pancreatic development, affecting either the whole pancreas (*IPF1*, *PTF1A*) or specifically beta cells (*GLIS3*) (3).

Pancreatic development is coordinated by a complex interplay of signaling pathways and transcription factors that determine early pancreatic specification as well as the later differentiation of exocrine and endocrine lineages (5, 6). The basic helix–loop–helix (bHLH) transcription factor NEUROD1 (also known as BETA2) plays an important role in the development of the endocrine pancreas. *NEUROD1* expression, along with *NEUROG3* and *INSM1*, specifies the endocrine lineage (7). *Neurod1* *-/-* mice fail to

develop mature islets, leading to ketosis-prone diabetes and death within the first few days of life (8).

Heterozygous loss-of-function mutations in *NEUROD1* have previously been identified as a very rare cause of maturity-onset diabetes of the young (MODY) and late-onset diabetes in humans, with only 5 families reported to date (9-12). We assessed the role of *NEUROD1* in PNDM and describe two unrelated probands with homozygous truncating *NEUROD1* mutations who have PNDM and similar neurological abnormalities.

RESEARCH DESIGN AND METHODS

This study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the local Ethics Committee and written informed consent was obtained from the parents/guardians of each patient.

Study population

We studied 44 probands with PNDM diagnosed before 6 months of age, who had been referred to the Molecular Genetics Laboratory at the Peninsula Medical School in Exeter, UK. Mutations in *KCNJ11*, *ABCC8*, *INS*, and *GCK* had been excluded. The relevant clinical information was obtained from the medical records.

NEUROD1 gene analysis

Genomic DNA was extracted from peripheral leukocytes using standard procedures. The single coding exon of *NEUROD1* was PCR-amplified in three overlapping fragments using specific primers for each amplicon tagged with 5' M13 tails to allow sequencing to be performed with a "universal" M13 primer (primers and conditions are available upon request). Single strand sequencing was carried out using standard methods on an ABI 3730 sequencer (Applied Biosystems, Warrington, UK). Sequences were compared to the published template (accession number NM_002500)

using Mutation Surveyor v3.20 (SoftGenetics, PA, USA). Sequence variants were tested for their presence in family members whenever DNA was available.

Homozygosity mapping

High-density single nucleotide polymorphism (SNP) genotyping was carried out on the Affymetrix human 10K Xba chip by Medical Solutions Nottingham (formerly GeneService), UK. Processing of genomic DNA was performed as per the Affymetrix protocol. In house Perl scripts were developed to automatically identify genomic homozygous segments, defined by at least 20 consecutive homozygous SNPs marking a region that exceeded 3 cM (13).

RESULTS

Molecular genetics

Two novel homozygous mutations in *NEUROD1*, a single base pair duplication (c.364dupG) and a 2-base pair CT deletion (c.427_428del), were identified in 2 unrelated probands. Both mutations result in a frameshift and a premature truncation of the C terminus of the expressed protein (p.Asp122Glyfs*12 and p.Leu143Alafs*55, respectively), leading to mutated proteins completely lacking the transactivation domain (Figure 1). These mutations had not been previously documented and were not present in 200 alleles from healthy unrelated individuals. No mutations were identified in the remaining 42 patients.

The two homozygous probands inherited the mutation from their heterozygous parents (Figure 2). In family A with the c.364dupG mutation, parents were known to be first cousins and, consistent with parental consanguinity, SNP genotyping analysis of the proband revealed a total genomic homozygosity value of 6.0% (13). The mutation-containing homozygous segment was the largest homozygous segment (46.6-Mb long)

and spanned 2q31.1-2q36.1 delimited by the SNPs rs726032 to rs724149. In contrast, in family B, the parents of the patient with the homozygous c.427_428del mutation were not known to be related and, in keeping with this, total genomic homozygosity value was very low (0.3%). However, the mutation in both parents was inherited on an extended haplotype of 10.4 Mb between positions Chr2q31.1-32.1 (SNPs: rs2884471-rs722385) suggesting that the mutation arose from a single common ancestor.

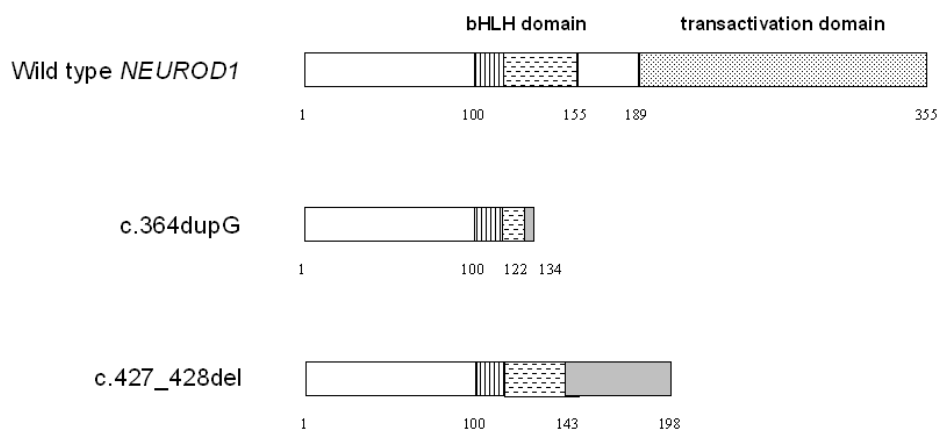


Figure 1. Schematic organization of NEUROD1 protein and effect of the two mutations on its structure. Numbers refer to the amino acids bordering the functional domains. Both mutations result in the generation of a truncated protein lacking the transactivation domain. The abnormal protein sequence between the frameshift and the termination codon is colored in grey.

Clinical features

The two probands were diagnosed with permanent diabetes within the first two months of life and had presented with intrauterine growth retardation (birth weights 1490 and 2230 g at 34 and 38 weeks of gestation, respectively) reflecting reduced insulin secretion *in utero*. They had no evidence of pancreatic exocrine dysfunction and normal pancreatic size on abdominal scanning. In addition to diabetes, they presented with a similar pattern of neurological abnormalities including moderate to severe developmental delay, profound sensorineural deafness, and visual impairment due to myopia and diffuse

despite having a normal BMI, and was treated with glicazide. In contrast, the father (also aged 33) had normal fasting (4-6 mmol/l) and postprandial (5-7 mmol/l) blood glucose levels on several occasions. In family B (c.427_428del mutation), the mother and father underwent standard oral glucose tolerance tests (aged 33 and 37 years) that confirmed normal glucose tolerance (6.2 and 4.8 mmol/L at 2 hours, respectively). No heterozygous family members in either family had any developmental delay or neurological features on clinical examination.

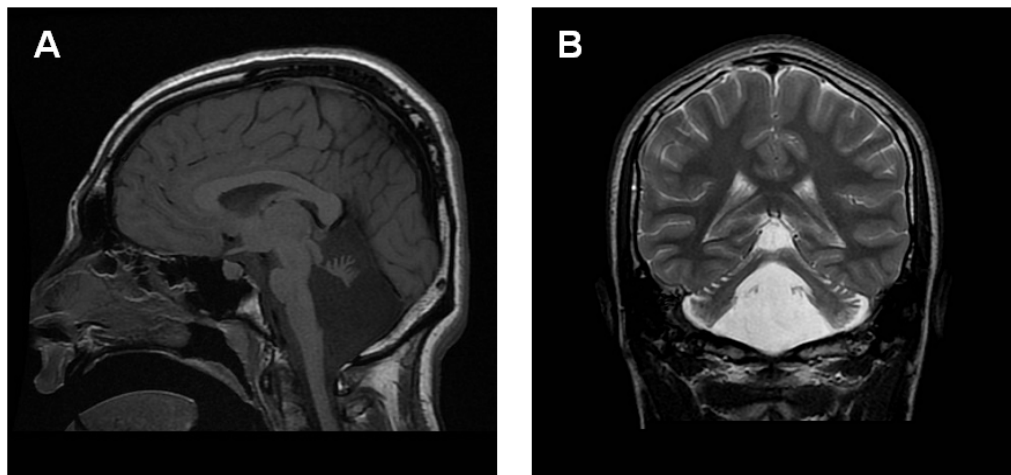


Figure 3. MRI of the brain in proband from Family A demonstrating the typical neuroimaging findings of *NEUROD1*-PNDM (Panel A: Sagittal T1-weighted image. Panel B: Coronal T2-weighted image). There is significant cerebellar hypoplasia particularly of cerebellar vermis inferiorly. Unusually the posterior fossa is well formed. Supratentorial midline structures and myelination are normal.

CONCLUSIONS

We report the first two cases of PNDM caused by homozygous mutations in *NEUROD1*. The patients with this novel autosomal recessive syndrome not only had early-onset permanent diabetes but also presented with developmental delay, cerebellar hypoplasia, and hearing and visual impairment. This is the 13th gene in which mutations have been described in patients with permanent neonatal diabetes.

	Case A (c.364dupG)	Case B (c.427_428del)
Gender	Female	Female
Country of origin	Pakistan	Hungary
Parental consanguinity	Yes (first cousins)	No
Birth information: - Gestational age (weeks) - Birth weight (grams) - Birth weight (SDS)	34 1490 -2.06	38 2230 -1.92
Diabetes mellitus: - Age at diagnosis (weeks) - Blood glucose (mmol/L) - Ketosis - C-peptide - Exocrine function Pancreas size	8 31.8 Yes N/A Normal Normal (MRI scan)	4 24.0 No Undetectable Normal Normal (CT scan)
Neurological Features: - Developmental delay - Cerebellar hypoplasia - Sensori-neural deafness - Visual impairment - Seizures	Yes Severe cerebellar hypoplasia (MRI), particularly of vermis Yes (Hearing aids; 80 dB loss) Severe myopia, diffuse retinal dystrophy (ERG reduced to approx. 25%) No epilepsy (two hypoglycemic seizures at 7 and 15 years).	Yes Severe cerebellar hypoplasia (MRI) Yes (Hearing aids) Moderate myopia, pigmental epithelial atrophy, enlarged fovea No epilepsy

Table 1. Clinical features of the two patients with homozygous *NEUROD1* mutations.

NEUROD1, a tissue-specific member of the basic helix-loop-helix (bHLH) family of transcription factors, is expressed in the developing pancreatic islets as well as in mature beta cells. It forms a heterodimer with the ubiquitous bHLH transcription factor E47 that binds to specific E-box motifs on specific target genes, including *INS*, *GCK*, and *ABCC8*, to regulate their expression (14-16). The two homozygous *NEUROD1* mutations both introduce a frameshift that result in truncated proteins lacking the transactivation domain which has been shown to be important for the interaction of NEUROD1 with its main coactivator, p300 (17). These are likely to have no biological activity as shown previously for a different frameshift mutation (c.616dupC, p.His206Profs*38) identified in a patient with *NEUROD1*-MODY (9).

Further evidence for the homozygous mutations in *NEUROD1* being etiological is that both unrelated patients have remarkably consistent, complex genetic syndrome whose clinical features are in keeping with the known expression and biology of this transcription factor (Table 1). Both unrelated patients have a remarkably consistent phenotype, with clinical features in keeping with the known expression and biology of this transcription factor and this provides further evidence for the homozygous mutations in *NEUROD1* being causative.

Both patients have neonatal diabetes but a normal pancreas on scanning and no evidence of an exocrine dysfunction. This is consistent with the central role of *NEUROD1* in islet development. Mice lacking *Neurod1* die shortly after birth from severe diabetic ketoacidosis (8). Histological examination of the *Neurod1*-deficient pancreas shows an impaired islet morphogenesis with a reduction in the number of endocrine cells, especially beta cells (8).

In addition to diabetes, our two patients presented with a similar pattern of neurological features, including developmental delay, cerebellar hypoplasia and visual and hearing impairment. This is in keeping with the abundant expression of *NEUROD1* in the developing and mature nervous system. Interestingly the initial *Neurod1*-null mice that rapidly died from diabetes had no obvious anatomic and histologic abnormalities of the brain (8). However it is possible to explore the role of *Neurod1* in the nervous system by rescuing *Neurod1*-null mice either by expressing a transgene encoding the mouse *Neurod1* gene under the insulin promoter (18) or by crossing the null mutation into a different genetic background to reduce the severity of the diabetes (19). The rescued *Neurod1*-null mice show a similar neuronal phenotype consisting of impaired balance, ataxic gait, circling, and swaying head movement as a result of impaired cerebellum development (18-20). Furthermore, rescued *Neurod1*-deficient mice have abnormal hearing and vision as a result of severe sensory neuronal defects in the inner ear and neural retina, respectively (20-22). The main feature seen in the mouse which was not present in our patients was epilepsy (19). The remarkable similarity between the

NEUROD1 deficient patients and the *Neurod1* deficient mice (Table 2) strongly supports a similar biological role of this transcription factor across species.

	Mouse model	Patients features
Endocrine pancreas	<ul style="list-style-type: none"> ▪ Early-onset ketosis-prone diabetes ▪ Failure of mature islets development ▪ Striking reduction in both beta and alpha cells 	<ul style="list-style-type: none"> ▪ Permanent neonatal diabetes
Exocrine pancreas	<ul style="list-style-type: none"> ▪ Postnatal-onset acinar cell polarity defects (indirect effect?) 	<ul style="list-style-type: none"> ▪ Normal
Enteroendocrine cells	<ul style="list-style-type: none"> ▪ Lack of secretin- and cholecystokinin-producing cells (remaining enteroendocrine cells normal) 	<ul style="list-style-type: none"> ▪ Not known
Cerebral cortex	<ul style="list-style-type: none"> ▪ Normal 	<ul style="list-style-type: none"> ▪ Normal
Dentate gyrus (hippocampus)	<ul style="list-style-type: none"> ▪ Seizures ▪ >95% decrease of granule cells 	<ul style="list-style-type: none"> ▪ No epilepsy
Cerebellum	<ul style="list-style-type: none"> ▪ Severe hypoplasia ▪ Impaired coordination, ataxia ▪ Decrease of granule cells 	<ul style="list-style-type: none"> ▪ Severe hypoplasia ▪ Ataxia
Retina	<ul style="list-style-type: none"> ▪ Blindness ▪ Decreased synapses, loss of outer nuclear layer 	<ul style="list-style-type: none"> ▪ Optic correction ▪ Retinal dysfunction
Inner ear	<ul style="list-style-type: none"> ▪ Deafness, imbalance ▪ Shortened cochlear duct, sensory epithelia abnormalities, degeneration of acoustic ganglions 	<ul style="list-style-type: none"> ▪ Sensorineural deafness

Table 2. Comparison of the major features seen in *Neurod1* deficient mice (8, 18-22) and *NEUROD1* deficient patients with homozygous *NEUROD1* mutations.

Homozygous mutations in *PTF1A*, which encodes another bHLH transcription factor, also cause a syndrome of neonatal diabetes and cerebellar hypoplasia/agenesis (23). However, in this condition the pancreatic phenotype is not limited to the islets as affected patients have pancreatic hypoplasia/aplasia. In keeping with the islets representing less than 1% of the endocrine pancreas, the size of the pancreas was found to be normal in our two patients with homozygous *NEUROD1* mutations. This suggests that shared developmental pathways are important during development in the pancreas and the cerebellum.

Although heterozygous loss-of-function mutations in *NEUROD1* have been previously identified as a very rare cause of diabetes in humans (9-12), diabetes was present in only one of four heterozygous mutation carrying parents. Their age at the time of the study ranged from 33 to 39 years, and does not exclude the possibility of developing diabetes later in life. In addition, incomplete penetrance has been described in some of the families with *NEUROD1*-diabetes (9). Homozygous mutations in other known MODY genes, namely *GCK* and *IPF1*, have been previously associated with isolated PNDM and isolated pancreatic agenesis, respectively (24, 25). We have shown that homozygous mutations in another MODY gene are also associated with a more severe phenotype, of neonatal diabetes.

In conclusion, homozygous mutations in *NEUROD1* constitute a rare novel autosomal recessive cause of neonatal diabetes with severe neurological abnormalities. This confirms the important role that *NEUROD1* plays in the development of both the pancreas and the nervous system in humans.

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CHAPTER 8

General discussion

This doctoral dissertation describes in detail various collaborative studies undertaken to increase the current knowledge on the aetiology of diabetes mellitus in infants and young children. The following discussion seeks integration between the various findings as such and in the context of the data available in the international literature. As a result, recommendations for future research are suggested.

8.1 UNRAVELING THE INTERCONNECTIONS BETWEEN MONOGENIC AND AUTOIMMUNE DIABETES IN PEDIATRICS

Insulin-dependent diabetes is a common disease in the paediatric setting. The vast majority of cases in western countries correspond to autoimmune type 1 diabetes. It is estimated to affect one in 500-1,000 children and adolescents, although its annual incidence rate varies widely between different countries, within countries, and between different ethnic populations (Craig et al, 2009). Monogenic diabetes is responsible for only 1-2% of diabetes cases and is often initially misdiagnosed as type 1 diabetes when insulin therapy is required. Identification of children with monogenic diabetes usually improves clinical care not only for themselves but also for their families. Genetic testing confirms the diagnosis, allows a definite classification of the specific subtype of monogenic diabetes, predicts the likely clinical course of the disease, defines risk for relatives, and may even determine the treatment of choice (Hattersley et al, 2009).

Differential diagnosis between type 1 diabetes and monogenic diabetes is relatively easy when patients present with additional extrapancreatic features suggesting a specific multisystemic disorder (Chapters 5 and 7 show a couple of examples). However, the distinction may become much more complicated when diabetes is the only clinical manifestation. Isolated insulin-deficient pediatric diabetes has been associated to a number of monogenic defects impairing β -cell function. They include heterozygous mutations in some of the classic MODY genes (mainly *HNF1A*, and to a lesser extent,

HNF4A) and several subtypes of neonatal and infancy-onset diabetes (mutations in *KCNJ11*, *ABCC8*, *INS* and *GCK*).

In recent years, different approaches to identify children with monogenic diabetes have been proposed, all of them based on the current knowledge of the natural history of type 1 diabetes. Type 1 diabetes is due to the autoimmune destruction of pancreatic insulin-producing β -cells as evidenced by a number of consistent findings, including the presence of circulating autoantibodies and activated T cells against target β -cell antigens in most patients (Knip et al, 2008; Arif et al, 2004), the positive clinical response to immune suppression (Bougnières et al, 1990; Herold et al, 2002), and the frequent coexistence of other autoimmune diseases (Anderson, 2002). Type 1 diabetes is a T-cell mediated organ-specific autoimmune disease so that circulating autoantibodies are considered to be just serological markers of the underlying pathologic process. They are present in over 90% of patients when hyperglycemia is first detected and a positive result at diabetes onset or shortly thereafter supports the diagnosis of autoimmune diabetes. Since proven autoimmune diabetes has been reported in patients without any pancreatic antibodies (Martin et al, 2001; Imagawa et al, 2001), a negative result, in contrast, does not exclude type 1 diabetes. Familial aggregation has been described in about 10% of cases of type 1 diabetes, although there is no recognizable pattern of inheritance (Hemminki et al, 2009). Inherited polygenic susceptibility to develop autoimmune type 1 diabetes is determined by at least 40 different genes (Barrett et al, 2009), with HLA genes showing the strongest association and accounting for up to 60% of the genetic susceptibility. Thus, 95% of patients with polygenic autoimmune type 1 diabetes have high-risk HLA haplotypes. However, these high-risk haplotypes are also found in ~40% of the general population even though most people will not develop the disease ever. Therefore, although the absence of a high-risk HLA genotype makes type 1 diabetes unlikely, its presence does not confirm the autoimmune aetiology on an individual basis (Marcovecchio et al, 2007).

8.1.1 Monogenic diabetes in late childhood and adolescence

In keeping with the aforementioned facts, mutations in *HNF1A* have been previously identified in adolescents and young adults initially thought to have type 1 diabetes. Most cases were selected for genetic testing on the basis of having a low-risk HLA haplotype, a strong family history of diabetes affecting at least 3 generations, or absent pancreatic antibodies (Yamada et al, 1997; Møller et al, 1998, Lambert et al, 2003; Kawasaki et al, 2000). Independently of the selection criterion used, a mutation in *HNF1A* was identified in 5-10% of probands tested. Similar studies for *HNF4A* have not been reported in the literature to date. We did not identify mutations in either gene in our study of 25 children with antibody-negative insulin-dependent diabetes (Chapter 2). However, this is not surprising since both *HNF1A* and *HNF4A* diabetes usually present at or after puberty and only two of our patients had been diagnosed with diabetes after 11 years of age. In order to better define the contribution of *HNF1A* and *HNF4A* mutations to diabetes presenting in peripubertal children and adolescents, prospective studies are needed. In this regard, the SEARCH study will provide valuable information in the near future (The SEARCH Writing Group, 2004). So far, a mutation in *HNF1A* or *HNF4A* has been found in about 5% of children with antibody-negative diabetes participating in the study (Gilliam et al, 2007; Hattersley & Ellard, unpublished data).

8.1.2 Monogenic diabetes in infants and young children

A well documented rise in the incidence of type 1 diabetes has been recently noted in many countries worldwide, especially in children under 5 years of age (Patterson CC et al, 2009). Since this diagnosis is often not considered by parents or health-care providers, such youngsters frequently present after several days or weeks of symptoms that were missed (Neu et al, 2003). Even though the diagnosis of diabetes is fairly rare among very young children, the clinical presentation of type 1 diabetes before 2 years of age is associated with severe metabolic decompensation, poorly preserved residual β -

cell function, strong humoral autoimmunity against islet cells and insulin, and strong HLA-defined disease susceptibility (Komulainen et al, 1999).

A large prospective population-based study on permanent diabetes diagnosed within the first year of life revealed the existence of two groups of patients based on age at onset, with a clear cut-off at around 180 days, and suggested that infants diagnosed before 6 months were unlikely to have classic autoimmune type 1 diabetes (Iafusco et al, 2002). In addition, low birthweight was more common in those patients, raising the possibility that the reduced insulin secretion was already present *in utero*. These findings were later confirmed by a second study, where 41% of patients diagnosed under 6 months had type 1 diabetes-associated high-risk HLA genotypes in contrast to 93% of infants diagnosed between 6 and 24 months (Edghill et al, 2006). Based on the fact that the prevalence of high-risk HLA was slightly lower in those diagnosed between 6 and 12 months of age (89%) compared with those presenting between 12 and 24 months of age (94%), it was hypothesized that there may be a few patients with non-autoimmune diabetes in the 6- to 12-month age range. On the other hand, persistent β -cell autoimmunity may appear slightly before the age of 6 months both in the offspring of mothers with type 1 diabetes (Martikainen et al, 1996) and in genetically susceptible individuals identified in the general population (Kimpimäki et al, 2001), suggesting that type 1 diabetes may very rarely manifest before 6 months of age. Hence, it is difficult to be certain of an absolute cut-off at 6 months between early-onset type 1 diabetes and monogenic diabetes of infancy. Most probably, genetic testing should be individually considered in patients diagnosed around that age (see below).

There are currently four known monogenic causes of isolated neonatal and infancy-onset diabetes. Recessively-acting mutations in *GCK* are uncommon and present within the first few weeks of life in newborns with severe intrauterine growth retardation (Chapter 4; Rubio-Cabezas & Hattersley, unpublished data). In contrast, mutations in the K_{ATP} channel genes (*KCNJ11* and *ABCC8*) and the proinsulin gene (*INS*) account for ~50% of cases of diabetes diagnosed under 6 months (Edghill et al, 2008). Heterozygous mutations in the *INS* gene have been reported in a few patients with diabetes presenting

beyond the first 6 months of life, most of whom were diagnosed before the age of 5 years (Støy et al, 2007; Edghill et al, 2008; Polak et al, 2008; Molven et al, 2008; Colombo et al, 2008; Støy et al, 2008; Bonfanti et al, 2009). In addition, *KCNJ11* mutations can also rarely present after 6 months (Mohamadi et al, 2009; Flanagan, Ellard & Hattersley, unpublished data). Both *INS* and *KCNJ11* mutations are rare in unselected patients with permanent diabetes diagnosed between 6 and 12 months of age, where they account for 2-3% of diabetes cases (Edghill et al, 2008; Støy et al, 2008; Flanagan, Ellard & Hattersley, unpublished data). Mutations in these two genes frequently appear *de novo* so family history of diabetes is often lacking. Moreover, ketosis or even ketoacidosis is frequent at diagnosis. Therefore, these patients cannot be clearly distinguished from those with early-onset type 1 diabetes just on clinical grounds. Our results (Chapter 2) have suggested that limiting genetic testing after the age of 6 months to patients with antibody-negative diabetes may increase the yield for identifying children with monogenic disease. To prove this hypothesis, a larger study looking at *KCNJ11*, *ABCC8* and *INS* in diabetic infants diagnosed between 6 and 12 months is currently ongoing in Exeter.

8.1.3 Monogenic autoimmune diabetes among diabetic children diagnosed within the first six months of life

As previously stated, polygenic type 1a diabetes accounts for the vast majority of autoimmune diabetes cases. It may or may not be associated to other autoimmune disorders, especially thyroiditis and celiac disease. In any case, the genetic predisposition is given by the combined effect of by many genes, each having a small effect individually (Concannon et al, 2009). The more risk alleles an individual has, the higher the risk of developing the disease. However, not all individuals with a high-risk genetic profile will present with diabetes. It is generally accepted that exposure to unknown environmental factors is also essential in the pathogenic process (Peng et al, 2006).

In addition to polygenic autoimmune diabetes, two rare monogenic autoimmunity syndromes including diabetes have been described (Ulmanen et al, 2005):

- Autoimmune polyglandular syndrome type 1 (APS1; also called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy [APECED]) is an autosomal recessive disorder secondary to mutations in the *AIRE* gene, encoding an autoimmune regulator protein. The patients develop multiple organ-specific autoimmune diseases, often starting in childhood or during teenage years (Ahonen et al, 1990). Hallmark symptoms include chronic *Candida* infection followed by autoimmune hypoparathyroidism and Addison's disease. At least two of the three aforementioned components should be present for diagnosis (Husebye et al, 2009), although many other associated immune-mediated disorders have been described. Diabetes is found in less than 20% of APECED patients and becomes prevalent toward middle age (Perheentupa et al, 2006).
- Mutations in *FOXP3* cause a monogenic X-linked recessive disorder known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (Torgerson et al, 2007). Diabetes is the commonest endocrine manifestation, affecting ~70% of patients, and presents within the first months of life. The outcome is very severe and usually results in early death. However, a few patients with a prolonged survival in whom diabetes was absent and the phenotype limited to autoimmune enteropathy have been reported (Powell et al, 1982; De Benedetti et al, 2006; Bacchetta et al, 2006).

IPEX syndrome is the only monogenic disorder known to produce autoimmune permanent neonatal diabetes. We have shown that the V408M mutation in *FOXP3* is associated with a relatively mild phenotype where very-early onset diabetes is the main clinical feature (Chapter 3). As a result, we have recommended that *FOXP3* should be sequenced in male patients with undiagnosed permanent neonatal diabetes who develop any further autoimmune disorders. Further research is needed to clarify the impact of *FOXP3* mutations in later onset autoimmune diabetes.

8.2 DISSECTING THE GENETIC ETIOLOGY OF PERMANENT NEONATAL DIABETES

Permanent neonatal diabetes is both clinically and genetically heterogeneous. Although it is meant to be a monogenic disorder in most patients, the underlying genetic defect remains unknown in ~40% of cases (Edghill et al, 2008), suggesting that other genetic causes are still to be identified. Among those patients with a known mutation, three genes (*KCNJ11*, *ABCC8* and *INS*) account for the majority of cases. All mutations reported to date in *KCNJ11* and *INS*, and most mutations in *ABCC8* are heterozygous and, hence, dominantly-acting. In contrast, some mutations in *ABCC8* and all mutations in *GCK* and other genes less frequently involved in PNDM are homozygous or compound heterozygous, indicating a recessive inheritance for the latter. The risk of developing one of these recessive subtypes of PNDM is therefore increased when parental consanguinity is present.

8.2.1 Parental consanguinity strongly influences the genetic aetiology in permanent neonatal diabetes

In order to assess whether known parental consanguinity actually influences the genetic aetiology in permanent neonatal diabetes, we studied a consecutive international series of 448 patients referred to Exeter for genetic testing (Rubio-Cabezas et al, 2009). Parental consanguinity was reported for 66 probands (15%). The three most common causes of PNDM (*KCNJ11*, *ABCC8* and *INS*) were sequenced in all patients. Further genetic investigation depended on the reported phenotype: in those patients with isolated PNDM, *GCK* was sequenced, whereas other genes were selectively tested in patients with additional extrapancreatic features. Even though mutations in *KCNJ11*, *ABCC8* and *INS* were the three most common genetic causes of PNDM in non-consanguineous families (33%, 11%, and 10% respectively), parental consanguinity was associated with a completely different genetic profile (Figure 1). Biallelic mutations in *EIF2AK3*, causing

Wolcott-Rallison syndrome, were the most common genetic cause of PNDM in our cohort (22%), followed by mutations in *INS* (13%), *GCK* (11%), and *ABCC8* (8%) mutations. Our findings on *EIF2AK3* and *INS* during this study have been displayed and discussed in detail in Chapters 4 and 5, respectively. Overall, K_{ATP} channel mutations (*KCNJ11* and *ABCC8*) accounted for 44% of non-consanguineous probands but only 10% of consanguineous cases, indicating that the number of patients with PNDM amenable to transition from insulin onto oral sulphonylureas is significantly lower when parents are related. Interestingly, the relative contribution of *INS* mutations to PNDM was similar in both groups. However, patients born to non-related parents had predominantly heterozygous coding mutations whereas patients from consanguineous pedigrees had homozygous coding and non-coding mutations.

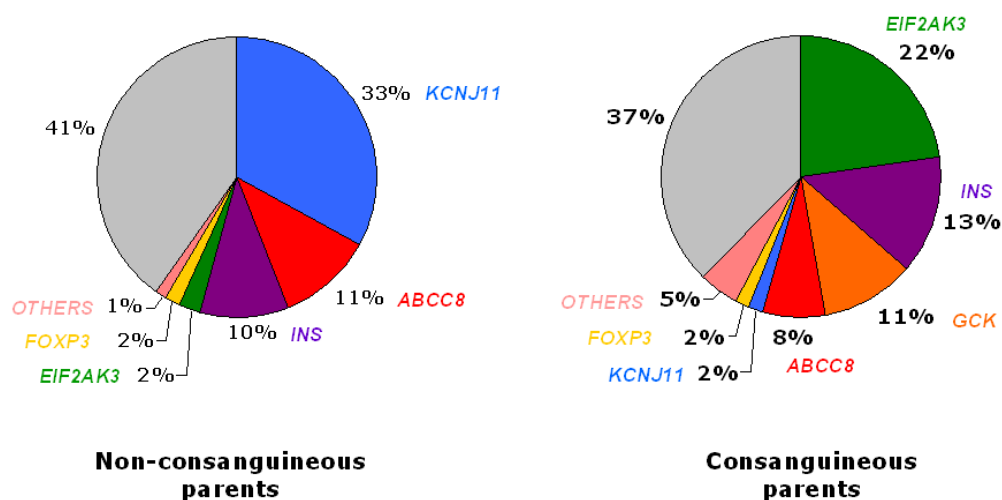


Figure 1. Influence of parental consanguinity on the genetic aetiology in permanent neonatal diabetes.

8.2.2 Novel insights into biology can be derived from human patients

The finding of recessively-acting mutations in the *INS* gene (Chapter 5) has had two main practical implications. On one side, it is the first time that different mutations in the same human gene produce the same disorder through two different mechanisms, namely β -cell apoptosis in heterozygous coding mutations and reduced or absent insulin

production in homozygous mutations. This is in keeping with the previous knowledge obtained from three different mouse models (Duvillié et al, 1997; Wang et al, 1999; Herbach et al, 2007). On the other hand, and maybe more importantly, we have identified a novel *cis* regulatory region within the *INS* promoter. Characterization of the unknown transcriptor factor(s) binding to this region to influence *INS* transcription level is currently ongoing.

8.2.3 Birth weight may be used as a clinical criterion to prioritise genetic testing in patients with permanent neonatal diabetes

Insulin is the main hormonal growth factor during both intrauterine and early postnatal life (Gicquel et al, 2006). It has repeatedly been reported that patients presenting with diabetes during the first 6 months of life tend to be small for gestational age at birth (Iafusco et al, 2002; Slingerland et al, 2006), which suggests that the insulin deficiency is already evident *in utero*. However, the degree of intrauterine growth retardation (IUGR) among patients with different subtypes of PNDM varies widely. For instance, patients unable to secrete any insulin both prenatally and postnatally due to pancreatic agenesis or biallelic mutations in the *GCK* or the *INS* genes show the more severe IUGR, with birth weights usually below –3 SDS for gestational age (Baumeister et al, 2005; Chapters 4 and 6). In contrast, birth weight in patients with heterozygous *INS* mutations or Wolcott-Rallison syndrome, although typically low, is usually above –2 SDS for gestational age (Edghill et al, 2008; Chapter 5). This is in keeping with their pathogenic mechanism involving progressive apoptosis of normally developed (and functioning) β -cells: despite the rapid decrease in insulin secretion postnatally leading to neonatal diabetes, prenatal insulin secretion is relatively spared and, therefore, intrauterine growth is not so much retarded. Interestingly, mutations in the K_{ATP} channel genes, although almost completely preventing insulin from being secreted (Pearson et al, 2006; Babenko et al, 2006), are associated with only slightly reduced birth weights (Edghill et al, 2008). There is currently no explanation for this unexpected finding.

8.2.4 Neonatal diabetes as a developmental disorder

Despite extensive research during the last few years, ~40% of permanent neonatal diabetes remains undiagnosed. So far, three different mechanisms leading to neonatal diabetes have been identified. A defective insulin secretion may be secondary to a functional impairment of normally developed β -cells, as in patients with activating mutations in *KCNJ11* or *ABCC8*, or homozygous loss-of-function mutations in *GCK* or *INS*. Alternatively, a progressive destruction of β -cells, either by apoptosis or autoimmune mechanisms, may reduce the number of β -cells and eventually cause neonatal diabetes, as in patients with heterozygous *INS* mutations, Wolcott-Rallison syndrome or IPEX syndrome. Finally, a reduced number of β -cells may also be the consequence of a disturbed pancreatic development. The latter mechanism has been involved in a minority of cases of permanent neonatal diabetes to date.

Pancreas development is coordinated by a complex interplay of signaling pathways and transcription factors that determine early pancreatic specification as well as the later differentiation of exocrine and endocrine lineages (Bonal et al, 2008; Oliver-Krasinski et al, 2008). Most of the known pancreatic transcription factors are expressed prior to or near the onset of pancreas development. Further, many of these factors regulate one another through positive feedback loops; as a result, earlier models of the transcriptional hierarchy have evolved into less linear schemes (Figure 2).

So far, only two of the genes from the list above have been implicated in the production of permanent neonatal diabetes in humans. Biallelic loss-of-function mutations in *PDX1*, also named *IPF1*, have been identified in three probands with isolated pancreatic aplasia (Stoffers et al, 1997; Schwitzgebel et al, 2003; Thomas et al, 2009). Homozygous *PTF1A* mutations cause pancreatic and cerebellar agenesis/hypoplasia (Sellick et al, 2004; Tutak et al, 2009). In addition, homozygous mutations in *GLIS3* were recently identified in patients with neonatal diabetes, congenital hypothyroidism and polycystic kidney disease (Senée et al, 2006). Although this gene had not been previously linked to pancreatic development, a recent study using knockout mice has

shown that it encodes a transcription factor required for proper differentiation of pancreatic endocrine cells (Watanabe et al, 2009). Somewhat surprisingly, no other transcription factor-encoding genes have been associated to permanent neonatal diabetes.

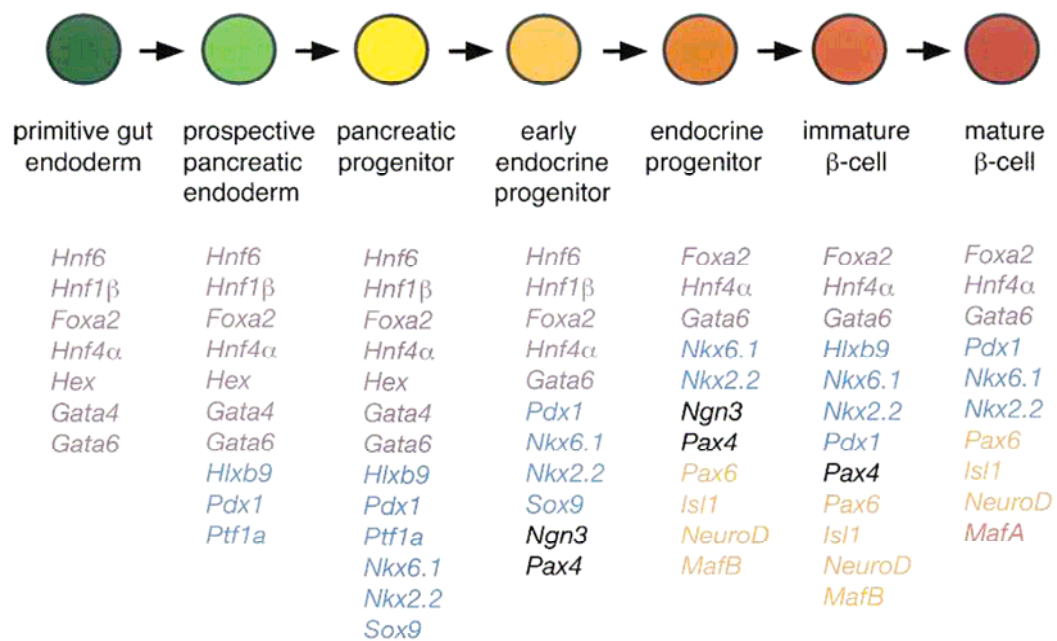


Figure 2. Transcription factor profile during β -cell formation from endodermal derivatives. Diagram indicates transcription factors expressed at each stage of differentiation. Factors initially expressed at a particular stage are color-coded as follows: gut endoderm (purple), pancreatic endoderm progenitor (blue), early endocrine progenitor (black), endocrine progenitor (orange), β -cell (red). [Reproduced from Oliver-Krasinski et al, 2008].

Based on the fact that heterozygous mutations in *GCK* and *IPF1* cause MODY, whereas homozygous or compound heterozygous mutations in the same genes produce permanent neonatal diabetes, we screened *NEUROD1* as a potential candidate gene for the latter. Heterozygous mutations had been previously described in a rare variant of MODY (Malecki et al, 1999). In keeping with its central role to pancreatic islet development and β -cell function, we have shown that biallelic loss-of-function mutations in *NEUROD1* cause a novel and rare subtype of neonatal diabetes (Chapter 7). The

clinical features of the two patients described strongly resemble the phenotype of the *NEUROD1* knockout mice.

Targeted gene disruption in mice has provided enormous insight into the role that transcription factors play during embryonic development of the pancreas. As many of these transcription factors are not pancreas-specific and also control other developmental processes elsewhere, extrapancreatic abnormalities in addition to diabetes have been described in most animal models. Therefore, a phenotype-driven approach may be useful to identify the genetic cause of undiagnosed permanent neonatal diabetes, especially in patients with a multisystemic syndrome. In addition to *NEUROD1*, we have recently identified the first patient with biallelic mutations in *NEUROG3* by using this candidate gene approach (Rubio-Cabezas et al, 2009; manuscript in preparation).

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CHAPTER 9

Summary and Conclusions

Resumen y conclusiones

SUMMARY

Monogenic diabetes is rare cause of diabetes in childhood and adolescence. Since most patients with monogenic diabetes presenting beyond the first 6 months of age are initially misdiagnosed as having type 1 diabetes, selection criteria are needed to identify those patients who may benefit most from genetic testing, especially when additional extrapancreatic features are lacking. Absence of pancreatic autoantibodies has been shown to increase the yield for identifying children with monogenic disease to the same extent than other previously suggested approaches, including the presence of a low-risk HLA genotype or strong family history of diabetes.

Despite recent advances in the understanding of the molecular bases of permanent neonatal diabetes, i.e. diabetes diagnosed within the first 6 months of life, approximately 40% of patients still remain molecularly undiagnosed. Development of autoimmune diabetes during the first 6 months of life is very rare and should raise the possibility of a monogenic autoimmunity disorder, especially in males who later present with any other immune-mediated condition. Among those patients with non-autoimmune permanent neonatal diabetes, the presence and quality of other multisystemic problems (for instance, skeletal dysplasia or liver dysfunction) informs the appropriate order in which genetic testing should be performed and, in case of previously undescribed syndromic combinations, may suggest potential novel candidate genes to test. Other clinical information such as birth weight or parental consanguinity is also very informative. Therefore, performing a complete and detailed medical history and physical examination in every single patient with neonatal diabetes should never be overlooked.

RESUMEN

La diabetes monogénica es una causa poco frecuente de diabetes mellitus durante la infancia y la adolescencia. Dado que la inmensa mayoría de los casos de diabetes monogénica que se manifiestan clínicamente después de los primeros 6 meses de edad son erróneamente diagnosticados como diabetes tipo 1, se necesitan criterios de selección para identificar aquellos pacientes que pueden beneficiarse de la realización de un estudio genético, sobre todo cuando no existen manifestaciones extrapancreáticas acompañantes. La ausencia de autoanticuerpos pancreáticos permite identificar a los niños con una posible diabetes monogénica con un rendimiento similar al de otros enfoques sugeridos previamente, como la presencia de un genotipo HLA de bajo riesgo para diabetes tipo 1 o antecedentes familiares de diabetes.

A pesar de los recientes avances en la comprensión de las bases moleculares de la diabetes neonatal permanente, es decir, la diabetes no transitoria diagnosticada en los primeros 6 meses de vida, aproximadamente el 40% de los pacientes permanecen sin diagnosticar desde el punto de vista genético. La presentación de diabetes autoinmune en los primeros 6 meses de vida es excepcional y debe hacer sospechar la posibilidad de un trastorno monogénico de autoinmunidad, especialmente en varones que más tarde desarrollan cualquier otra enfermedad autoinmune. Entre los pacientes con diabetes neonatal permanente no autoinmune, la presencia y el tipo de manifestaciones clínicas en otros órganos o sistemas (por ejemplo, displasia esquelética o disfunción hepática), ayudan a decidir el orden apropiado en el que se deben realizar los estudios genéticos y, en caso de combinaciones sindrómicas no descritas anteriormente, pueden sugerir nuevos genes candidatos para ser estudiados. Otros datos clínicos, como el peso al nacer o la consanguinidad parental son también muy informativos. Por lo tanto, la importancia de realizar una historia clínica y un examen físico completos y detallados en todos los pacientes con diabetes neonatal no debe menospreciarse.

CONCLUSIONS

- I. Measurement of pancreatic autoantibodies could be a useful strategy to identify candidates who may benefit from genetic testing for monogenic diabetes among children with insulin-dependent diabetes.
- II. Young children with antibody-negative diabetes should be screened for mutations in the *INS* gene, especially when progression to complete insulin deficiency is slow, irrespective of the presence of family history of diabetes.
- III. Mutations in *FOXP3* should be excluded in male patients with undiagnosed permanent neonatal diabetes who develop another immune-mediated disease later in life.
- IV. Presence of parental consanguinity strongly influences the genetic aetiology in permanent neonatal diabetes and could be used as a selection criterion to prioritise genetic testing. Birth weight may also help in the decision-making process.
- V. Biallelic mutations in *EIF2AK3*, *INS* and *GCK* are the three most common genetic causes of permanent neonatal diabetes among children born to consanguineous parents. In this setting, patients are much less likely to successfully transfer from insulin onto oral sulfonylureas.
- VI. A phenotype-driven approach might be used to identify new genetic causes of permanent neonatal diabetes, especially in patients with additional extrapancreatic features.

CONCLUSIONES

- I. La determinación de anticuerpos antipancreáticos podría ser una estrategia útil para seleccionar, de entre los niños con diabetes insulino-dependiente, aquellos que podrían beneficiarse de la realización de un estudio genético para confirmar una posible diabetes monogénica.
- II. Es recomendable descartar la existencia de mutaciones en el gen *INS* en los lactantes y niños pequeños con diabetes mellitus pero sin autoanticuerpos, especialmente cuando la progresión a la deficiencia completa de insulina es lenta, independientemente de la presencia de antecedentes familiares de diabetes.
- III. Se deben excluir mutaciones en *FOXP3* en los varones con diabetes neonatal permanente que desarrollan posteriormente cualquier otra enfermedad potencialmente autoinmune.
- IV. La presencia de consanguinidad parental influye fuertemente sobre la causa genética de la diabetes neonatal permanente y puede ser utilizada como criterio de selección para priorizar los posibles estudios genéticos. El peso al nacer también puede ayudar en este proceso de decisión.
- V. Las mutaciones bialélicas en *EIF2AK3*, *INS* y *GCK* son las tres causas genéticas más comunes de diabetes neonatal permanente en los niños con antecedentes de consanguinidad. En este contexto, los pacientes tienen *a priori* menos posibilidades de ser tratados con sulfonilureas por vía oral.
- VI. Es posible identificar nuevas causas genéticas de diabetes neonatal permanente mediante un enfoque basado en el fenotipo de los pacientes, especialmente en aquellos que asocian manifestaciones extrapancreáticas adicionales.

